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(54) Title: FUSION PROTEINS FOR DELIVERY OF GDNF TO THE CNS

(57) Abstract:

FUSION PROTEINS FOR DELIVERY OF GDNF TO THE CNS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]   This application in a continiation-in-part of U.S. Patent Application No. 11/245,546 filed
October 7, 2005. This application also claims priority to U.S. Provisional Patent Application serial
No. 60/990,290 filed November 26, 2007, the contents of which are incorporated herein by
reference in their entirety.

BACKGROUND OF THE INVENTION

[0002]   Neurological disorders represent a major cause of mortality and disability worldwide.
Despite extensive progress, current treatment options remain limited in some aspects. One major
reason for this limitation is that the brain is unique in allowing only select access to molecules.
While this is a useful protective mechanism, it also means that many potentially beneficial
molecular entities do not have access to the central nervous system (CNS), and thus are unable to
exert a therapeutic effect in many neurological disorders or other conditions of the CNS. The
present invention represents an advance in providing accessibility of the CNS for molecular entities
whose ability to cross the blood brain barrier is limited.

SUMMARY OF THE INVENTION

[0003]   In one aspect, the invention provides a composition comprising a neurotherapeutic peptide
comprising an amino acid sequence which is at least 80% (e.g., 95%) identical to the amino acid
sequence of mature human GDNF (SEQ ID NO:52) covalently linked to a structure that is capable
of crossing the blood brain barrier (BBB) (e.g., an antibody). In some embodiments, the structure
that is capable of crossing the BBB crosses the BBB on an endogenous BBB receptor. In some
embodiments, the endogenous BBB receptor is the insulin receptor, transferrin receptor, leptin
receptor, lipoprotein receptor, and the IGF receptor. In some embodiments, the structure that is
capable of crossing the BBB is a monoclonal antibody. In some embodiments, the monoclonal
antibody is a chimeric monoclonal antibody. In one embodiment, the chimeric antibody contains
sufficient human sequences to avoid significant immunogenic reaction when administered to a
human. In some embodiments, the above-mentioned neurotherapeutic peptide is covalently linked
at its amino terminus to the carboxy terminus of the heavy chain of the MAb. In one embodiment,
the neurotherapeutic peptide of the above-mentioned composition comprises the amino acid
sequence of mature human GDNF.

[0004]   In a further aspect provided herein is a composition for treating a neurological disorder
comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80%
identical to the amino acid sequence of mature human GDNF covalently linked to an
immunoglobulin that is capable of crossing the blood brain barrier, wherein the composition is
capable of crossing the BBB in an amount that is effective in treating the neurological disorder. In

some embodiments provided herein is a mammalian cell comprising the just-mentioned composition.

[0005]   In another aspect provided herein is a method of transport of a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52) from the peripheral circulation across the BBB in an effective amount, comprising peripherally administering to an individual the GDNF covalently attached to a structure that crosses the BBB, under conditions where the agent covalently attached to a structure that crosses the BBB is transported across the BBB in an effective amount.

[0006]   In yet another aspect provided herein is a method for treating a CNS disorder in an individual comprising peripherally administering to the individual an effective amount of a composition comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% (e.g., 95%) identical to the amino acid sequence of human GDNF covalently attached to a structure capable of crossing the BBB. In one embodiment, the neurotherapeutic peptide comprises the amino acid sequence of human GDNF. In some embodiments, the administering is oral, intravenous, intramuscular, subcutaneous, intraperitoneal, rectal, transbuccal, intranasal, transdermal, or inhalation administration. In some embodiments, the CNS disorder is an acute CNS disorder (e.g., spinal cord injury, brain injury focal brain ischemia and global brain ischemia). In other embodiments, the CNS disorder is a chronic CNS disorder (e.g., a chronic neurodegenerative disease, drug addiction, or alcohol addiction). In some embodiments, the chronic neurodegenerative disease is Parkinson's disease or a motor neuron disease (e.g., amyotrophic lateral sclerosis). In some embodiments, the individual to be treated is administered about 1 to about 100 mg of the composition used in above-mentioned method.

[0007]   In yet another aspect provided herein is a composition comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of human GDNF covalently linked to an immunoglobulin, wherein the neurotherapeutic peptide has a serum half-life that is an average of at least about 5-fold greater than the serum half-life of the neurotherapeutic peptide alone. In some embodiments, the immunoglobulin is an antibody to an endogenous BBB receptor (e.g., the insulin receptor, transferrin receptor, leptin receptor, lipoprotein receptor, or the IGF receptor). In one embodiment, the neurotherapeutic peptide comprises the amino acid sequence of human GDNF.

[0008]   In a further aspect provided herein is a method for treating substance abuse in an individual, comprising administering to the individual an effective amount of a composition comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52) covalently attached to a structure capable of crossing the BBB.

INCORPORATION BY REFERENCE

[0009]   All publications and patent applications mentioned in this specification are herein
incorporated by reference to the same extent as if each individual publication or patent application
was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]   The novel features of the invention are set forth with particularity in the appended claims.
A better understanding of the features and advantages of the present invention will be obtained by
reference to the following detailed description that sets forth illustrative embodiments, in which the
principles of the invention are utilized, and the accompanying drawings of which:

[0011]   **Figure 1.** Diagram showing genetic engineering of a eukaryotic expression vector
encoding a fusion gene comprised of the variable region of the heavy chain (VH) of the chimeric
HIRMAb, a genomic fragment encoding the constant region of human IgG1, which is comprised of
4 regions (CH1, hinge, CH2, and CH3), and the cDNA for the BDNF variant (vBDNF).
Transcription of the gene is driven by the human IgG1 promoter (PRO). This vector produces the
heavy chain (HC) of the fusion protein.

[0012]   **Figure 2.** Diagram showing genetic engineering of a bacterial expression plasmid
encoding vBDNF cDNA with modified 5'- and 3'-linkers.

[0013]   **Figure 3.** Ethidium bromide stained agarose gels showing size of various constructs that
are intermediates in construction of a tandem vector that produces the fusion protein. (A) Lanes 1-2:
plasmid from figure 2 digested with NruI showing 0.4 kb vBDNF and 3.5 kb vector backbone. Lane
3: MW size standards ranging from 1.4-0.1 kb. Lane 4: MW size standards ranging from 23-0.6 kb.
(B) Lane 1: the 0.4 kb vBDNF cDNA is produced by the polymerase chain reaction (PCR) using
cDNA reverse transcribed from polyA+ RNA isolated from human U87 glioma cells; the PCR
primer sequences are given in Table 2. Lanes 2 and 3: same MW size standards as shown in panel
A. (C) lane 1: clone 416 following digestion with NheI and BamHI; lane 2: negative clone; lane 3:
clone 400 following digestion with NheI and BamHI: lanes 4 and 5: same MW size standards as
shown in panel A. (D) PCR fragments of DNA encoding fusion protein HC (lane 1) and LC (lane
2); lanes 3-4: same MW size standards as shown in panel A. (E) lanes 1-4: 4 different but identical
copies of clone 422a following digestion with NheI, showing release of 0.4 kb fusion protein HC
variable region (VH) cDNA; lanes 5-6: same MW size standards as shown in panel A. (F) lanes 1-
4: 5 different but identical copies of clone 423a following digestion with EcoRV and BamHI,
showing release of 0.7 kb entire LC cDNA; lanes 5-6: same MW size standards as shown in panel
A. (G) Restriction endonuclease mapping of tandem vector (Figure 12) with PvuI (lane 1), and
EcoRI-HindIII (lane 2). PvuI (single cut) produced the expected linear DNA band of ~11 kb.
Digestion with EcoRI and HindIII releases both the fusion protein light chain (i.e. 1.8 kb) and
DHFR (i.e. 1.5 kb) expression cassettes. The ~8 kb band represents the backbone vector with the
fusion protein heavy chain expression cassette; lanes 3-4: same MW size standards as shown in
panel A, albeit in reverse order.

[0014]   **Figure 4.** Nucleotide (SEQ ID NO: 21) and amino acid (SEQ ID NO: 22) sequence of fusion site between carboxyl terminus of the fusion protein HC and the amino terminus of the vBDNF. The 3-amino acid linker between the HIRMAb HC and the vBDNF is shown, as well as the new stop codon at the carboxyl terminus of vBDNF.

[0015]   **Figure 5.** Nucleotide sequence (SEQ ID NO: 23) of fusion protein HC gene cloned into plasmid 416. Italics: human IgG1 constant region introns; bold font: human IgG1 exon sequence; underline font: vBDNF.

[0016]   **Figure 6.** Amino acid sequence (SEQ ID NO: 24) of the fusion protein HC. The 19 amino acid signal peptide is underlined, as is the 3-amino acid linker between the CH3 region and the vBDNF. The N-linked glycosylation consensus sequence within CH2 is underlined.

[0017]   **Figure 7.** The amino acid sequence (SEQ ID NO: 25) of the different domains of the fusion protein HC are shown.

[0018]   **Figure 8.** Diagram showing production of the intronless eukaryotic expression vector, clone 422a, which encodes the fusion protein HC. The fusion protein HC cDNA was produced by PCR from cDNA generated by reverse transcriptase of RNA isolated from myeloma cells transfected with clone 416.

[0019]   **Figure 9.** (A) Nucleotide sequence (SEQ ID NO: 26) of the fusion protein HC cDNA inserted in clone 422a. (B) (SEQ ID NOS 27 & 28) Amino acid sequence of the fusion protein HC that is deduced from the nucleotide sequence shown in panel A. The sequence of the signal peptide is underlined.

[0020]   **Figure 10.** Diagram showing production of the intronless eukaryotic expression vector, clone 423a, which encodes the fusion protein LC. The fusion protein LC cDNA was produced by PCR from cDNA generated by reverse transcriptase of RNA isolated from myeloma cells transfected with an expression vector producing the LC gene that was derived from chromosomal fragment encoding intron/exon sequence of the human kappa LC gene with the VL of the chimeric HIRMAb LC.

[0021]   **Figure 11.** (A) Nucleotide sequence (SEQ ID NO: 29) of the fusion protein LC cDNA inserted in clone 423a. (B) (SEQ ID NOS 30 & 31) Amino acid sequence of the fusion protein LC that is deduced from the nucleotide sequence shown in panel A. The sequence of the signal peptide is underlined.

[0022]   **Figure 12.** Diagram showing the construction of a tandem vector encoding the HC and LC genes of the fusion protein. The TV was engineered from the cDNA expression vectors, clones 422a and 423a, for the HC and LC, respectively, as well as from a bacterial expression plasmid encoding the expression cassette for mouse DHFR.

[0023]   **Figures 13A and 13B.** Nucleotide sequence (SEQ ID NO: 32) of the fusion protein HC gene and LC gene, and the DHFR genes incorporated in the tandem vector.

[0024]   **Figure 14.** Deduced amino acid sequence of the fusion protein HC based on tandem vector nucleotide sequence analysis (SEQ ID NOS 33 & 34). The signal peptide sequence is underlined.

[0025]   **Figure 15.** Deduced amino acid sequence of the fusion protein LC based on tandem vector nucleotide sequence analysis (SEQ ID NO 35 & 36). The signal peptide sequence is underlined.

[0026]   **Figure 16.** Deduced amino acid sequence of the DHFR based on tandem vector nucleotide sequence analysis (SEQ ID NO 37 & 38).

[0027]   **Figure 17.** Viable and total cell density of CHO cells in bioreactor maintained continuously for 50 days; the CHO cells had been stably transfected with the tandem vector encoding the fusion protein.

[0028]   **Figure 18.** Structure of fusion protein, a bi-functional molecule that both (a) binds to the human BBB human insulin receptor (HIR) to enable transport across the BBB from blood, and (b) binds to the trkB on neurons to induce neuroprotection.

[0029]   **Figure 19.** Correlation of 2 different 'sandwich' immunoassays, where the secondary antibody is either directed against the Fc region of human IgG1 (x-axis) or against human BDNF (y-axis). The primary antibody in either assay is directed against the human kappa light chain. The measured level of fusion protein in CHO cell conditioned medium is the same whether the anti-Fc or the anti-BDNF antibody is used.

[0030]   **Figure 20.** Reducing (left) and non-reducing (right) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of chimeric HIRMAb and fusion protein. Under reducing conditions, the size of the light chain, 30 kDa, is identical for chimeric HIRMAb and the fusion protein; the size of the heavy chain of fusion protein is about 15 kDa larger than the chimeric HIRMAb heavy chain, owing to the presence of the BDNF. Under non-reducing conditions, the chimeric HIRMAb and the fusion protein migrate as single hetero-tetrameric species with molecular weights of 180 and 200 kDa, respectively.

[0031]   **Figure 21.** (Left panel) Western blot with anti-human IgG primary antibody. The size of the heavy chain of the fusion protein and the chimeric HIRMAb is 64 kDa and 50 kDa, respectively, and the size of the light chain for either the fusion protein or the chimeric HIRMAb is 25 kDa. (Right panel) Western blot with anti-human BDNF antibody, which reacts with either fusion protein or BDNF, but not with chimeric HIRMAb. MW standards (STDS) are shown on the right side.

[0032]   **Figure 22.** Isoelectric focusing (IEF) of isoelectric point (pI) standards (lane 1), chimeric HIRMAb (lanes 2 and 4), BDNF (lane 3), and fusion protein (lane 5). Whereas BDNF is highly cationic with a pI>10, the pI of the fusion protein approximates the pI of the chimeric HIRMAb, which is about 8.5, and close to the theoretical pI of the fusion protein.

[0033]   **Figure 23.** (A) Outline for human insulin receptor (HIR) competitive ligand binding assay (CLBA). The HIR extracellular domain (ECD) is bound by the $[^{125}I]$-labeled murine HIRMAb, and this binding is competitively displaced by either the chimeric HIRMAb or the fusion protein, as

shown in Panel B. (B) Displacement of binding of [^{125}I]-labeled murine HIRMAb to the HIR ECD by either chimeric HIRMAb or fusion protein. The affinity of the chimeric HIRMAb to the HIR ECD is high, and the affinity of the fusion protein for the HIR ECD is not significantly different from that of the chimeric HIRMAb. These results show that the fusion of the vBDNF to the carboxyl terminus of the chimeric HIRMAb heavy chain does not impair binding of the fusion protein to the HIR.

[0034]   **Figure 24.** (A) Design of trkB competitive ligand binding assay (CLBA). The advantage of the PEG linker is that this modification eliminates the high non-specific binding (NSB) of the cationic BDNF to the ELISA wells, which gives an assay with a high signal/noise ratio. The binding of the BDNF-PEG^{2000}-biotin to the trkB ECD was detected with a peroxidase system using avidin and biotinylated peroxidase. (B) The binding of the BDNF-PEG^{2000}-biotin to the trkB ECD is competitively displaced by recombinant BDNF. This binding data was analyzed by non-linear regression analysis to yield the K_i of BDNF binding, 3.5 ± 1.3 pmol/well and the NSB parameter. (C) The binding of the BDNF-PEG^{2000}-biotin to the trkB ECD is competitively displaced by the fusion protein. This binding data was analyzed by non-linear regression analysis to yield the K_i of fusion protein binding, 2.2 = 1.2 pmol/well, which is not significantly different than the K_i for native BDNF. These data show that the affinity of the fusion protein for the trkB receptor is equal to that of native BDNF.

[0035]   **Figure 25.** (A) Design of hypoxia-reoxygenation neuroprotection assay in human neural SH-SY5Y cells. Exposure of the cells to retinoic acid for 7 days causes an up-regulation in the gene expression of trkB, the BDNF receptor. (B) Neuroprotection assay based on the measurement of mitochondrial respiration with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). The maximal neuroprotection is established with 4 nM BDNF, and 4 nM fusion protein yields a comparable level of neuroprotection in human neural cells. The MTT level does not return to that of non-hypoxic cells, because only about 50% of the cells induce trkB in response to retinoic acid.

[0036]   **Figure 26.** (A) Light micrograph of capillaries isolated from human brain, used as an in vitro model system of the human BBB. (B) Radio-receptor assay of binding of [^{3}H]-fusion protein to the HIR on the human BBB; the binding is self-inhibited by unlabeled fusion protein. Fitting the saturation data to a Scatchard plot with a non-linear regression analysis yields the binding parameters: K_D= 0.55 ± 0.07 nM, B_{max} = 1.35 ± 0.10 pmol/mg$_p$.

[0037]   **Figure 27.** Pharmacokinetics and brain uptake of fusion protein in the adult Rhesus monkey. (A) The serum concentration of [^{3}H]-fusion protein, or [^{125}I]-murine HIRMAb, is plotted vs. time after a single intravenous injection of either protein in anesthetized adult Rhesus monkeys. (B) The serum radioactivity that is precipitable by trichloroacetic acid (TCA) is plotted vs time after a single intravenous injection of either [^{3}H]-fusion protein in the anesthetized adult Rhesus monkey, or [^{3}H]-BDNF in the anesthetized adult rat. (C) Capillary depletion analysis of brain distribution at 180 minutes after a single intravenous injection of either [^{3}H]-fusion protein, or [^{3}H]-mouse IgG2a,

in the anesthetized adult Rhesus monkey. (D) Primate brain concentrations of fusion protein at 180 minutes after an intravenous injection of 373 µg fusion protein, as compared to the endogenous primate brain concentration of BDNF.

[0038]   **Figure 28.** Schematic illustration of the protein formed by fusion of the amino terminus of the mature GDNF to the carboxyl terminus of the CH3 region of the heavy chain of the chimeric HIRMAb. The fusion protein is a bi-functional molecule: the fusion protein binds the HIR, at the BBB, to mediate transport into the brain, and binds the GFRα1, to mediate GDNF biologic effects in brain behind the BBB.

[0039]   **Figure 29.** (A) Ethidium bromide stain of agarose gel of human GDNF cDNA (lane 1), which was produced by PCR from cDNA produced by reverse transcription of RNA from human U87 glial cells, and GDNF-specific ODN primers (Table 6). Lane 2: DNA sizing standards. (B) Genetic engineering of pHIRMAb-GDNF, the eukaryotic expression plasmid encoding the fusion protein of GDNF and the heavy chain (HC) of the chimeric HIRMAb. The fusion gene is 5'-flanked by the cytomegalovirus (CMV) promoter and 3'-flanked by the bovine growth hormone polyA (pA) sequence.

[0040]   **Figure 30.** Reducing SDS-PAGE and Coomasie blue staining of protein A affinity purified, COS-cell chimeric HIRMAb and the HIRMAb-GDNF fusion protein. Both are purified to homogeneity and are comprised of a heavy chain and a light chain. The molecular weight (MW) of the heavy chain (HC) of the HIRMAb-GDNF fusion protein is about 15 kDa larger than the MW of the HC of the chimeric HIRMAb, owing to fusion of the 15 kDa GDNF.

[0041]   **Figure 31.** Western blot with either anti-human (h) IgG primary antibody (left) or an anti-human GDNF primary antiserum (right). The immunoreactivity of the COS cell derived HIRMAb-GDNF fusion protein is compared to the chimeric HIRMAb and to recombinant GDNF. Both the HIRMAb-GDNF fusion protein and the HIRMAb have identical light chains on the anti-hIgG Western. The HIRMAb-GDNF fusion heavy chain reacts with both the anti-hIgG and the anti-human GDNF antibody, whereas the HIRMAb heavy chain only reacts with the anti-hIgG antibody. The size of the HIRMAb-GDNF fusion heavy chain, 70 kDa, is about 15 kDa larger than the size of the heavy chain of the HIRMAb, owing to the fusion of the 15 kDa GDNF to the 55 kDa HIRMAb heavy chain.

[0042]   **Figure 32.** Binding of either the COS cell-derived chimeric HIRMAb or the HIRMAb-GDNF fusion protein to the HIR extracellular domain (ECD) is saturable. The ED50 of HIRMAb-GDNF binding to the HIR ECD is comparable to the ED50 of the binding of the chimeric HIRMAb.

[0043]   **Figure 33.** (A) Outline of GFRα1 receptor binding assay. The GFRα1:Fc fusion protein is captured by a mouse anti-human (MAH) Fc antibody. The GDNF, or HIRMAb-GDNF fusion protein, binds to the GFRα1, and this binding is detected with a goat anti-GDNF antibody and a rabbit anti-goat (RAG) antibody conjugated to alkaline phosphatase (AP). (B) Binding of either the

COS cell derived chimeric HIRMAb or the HIRMAb-GDNF fusion protein to the GFRα1
extracellular domain (ECD) is saturable. The ED50 of HIRMAb-GDNF binding to the GFRα1
ECD is comparable to the ED50 of the binding of recombinant GDNF. There is no binding to the
GFRα1 by the chimeric HIRMAb.

[0044]   **Figure 34.** (A) Binding by either GDNF or the HIRMAb-GDNF fusion protein to the
GFRα1 on the cell membrane of c-ret kinase transfected human neural SK-N-MC cells activates the
tyrosine hydroxylase (TH) 5'-flanking sequence (FS), which drives luciferase gene expression. (B)
Both GDNF and the COS cell-derived HIRMAb-GDNF fusion protein activate luciferase gene
expression in a saturable manner, with ED50 values comparable to GFRα1 binding (Figure 33B).
There is no activation of luciferase gene expression by the chimeric HIRMAb.

[0045]   **Figure 35.** (A) Effect of intra-cerebral injection of the HIRMAb-GDNF versus saline on
stroke volume in rats. Serial coronal sections of rat brain at 24 hours after permanent MCAO and
intra-cerebral saline (B) or HIRMAb-GDNF fusion protein (C) are stained with 2% 2,3,5-
triphenyltetrazolium chloride (TTC). A representative stain is shown for 1 rat from each treatment
group. In these inverted grayscale images, the infarcted brain is black, and the healthy brain is
white. Most rostral section is top and most caudal section is bottom.

[0046]   **Figure 36.** Domain structure and amino acid sequence of the heavy chain (HC) of the
HIRMAb-GDNF fusion protein. The polypeptide is comprised of a signal peptide, followed by the
variable region of the heavy chain (VH) of the chimeric HIRMAb. The 3 CDRs and 4 FRs are
shown. The VH region is followed by the human IgG1 constant region, which is comprised of the
CH1, hinge, CH2, and CH3 domains, followed by a 2-amino acid (Ser-Ser) linker, followed by the
mature human GDNF. The single N-linked glycosylation site within the CH2 region, and the 2 N-
linked glycosylation sites within the GDNF are underlined with the asparagine (N) residues in bold
font. Cysteine (C) residues, which form inter-chain disulfide bonds are shown, and include a
linkage between the HC hinge region and the light chain (LC), the HC hinge region and the paired
HC, the HC CH2 region and the paired HC, and the GDNF-GDNF linkage.

[0047]   **Figure 37.** Tandem vector (TV) encoding multiple genes on a single piece of DNA. The
GDNF cDNA, produced by PCR, is subcloned into the HpaI site of the universal tandem vector
(UTV) to generate the HIRMAb-GDNF TV, which allows for expression in CHO cells of the fusion
heavy chain (HC) gene, the light chain (LC) gene, and the dihydrofolate reductase (DHFR) gene.

[0048]   **Figure 38.** Domain structure of the light chain of the HIRMAb.

[0049]   **Figure 39.** Western blot with a primary antibody against either human IgG (A) or human
GDNF (B). The CHO-derived chimeric HIRMAb and the CHO-derived HIRMAb-GDNF fusion
protein are applied in panel A, and the HIRMAb, the HIRMAb-GDNF fusion protein, and GDNF
are applied in panel B. The migration of molecular weight standards is shown in lane 1 of panel A.

[0050]   **Figure 40.** The affinity of the CHO-derived HIRMAb and the CHO-derived HIRMAb-
GDNF fusion protein for binding to the extracellular domain of the human insulin receptor (HIR) is

measured in this ELISA format. The concentration of antibody that causes 50% saturation of binding, the ED50, was determined by non-linear regression analysis. The 2 proteins have the same affinity for the HIR, indicating fusion of GDNF to the C-terminus of the heavy chain of the HIRMAb does not affect binding to the HIR.

[0051]   **Figure 41**. Size exclusion high performance liquid chromatography of the HIRMAb-GDNF fusion protein shows the absence of aggregates in the CHO-derived protein.

[0052]   **Figure 42**. Native polyacrylamide gel electrophoresis of the HIRMAb, control human IgG1, or the HIRMAb-GDNF fusion protein shows the absence of aggregates in the CHO-derived fusion protein.

[0053]   **Figure 43**. (A) Light micrograph of isolated human brain capillaries, used as an in vitro model system of the human BBB. (B) Binding of the ^{3}H-HIRMAb-GDNF fusion protein to the isolated brain capillaries is inhibited by the murine HIRMAb. The component of HIRMAb-GDNF uptake by the capillaries that is resistant to HIRMAb inhibition represents endocytosed fusion protein.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

II. Definitions

III. The blood brain barrier

      A. Transport systems

      B. Structures that bind to a blood brain barrier receptor-mediated transport system

IV. Agents for transport across the blood brain barrier

      A. Neurotrophins

      B. Brain-derived neurotrophic factor

      C. Glial-derived neutrotrophic factor

V. Compositions

VI. Nucleic acids, vectors, cells, and manufacture

      A. Nucleic acids

      B. Vectors

      C. Cells

      D. Manufacture

VII. Methods

VIII. Kits

Abbreviations

AA	amino acid
ALS	amyotrophic lateral sclerosis
AP	alkaline phosphatase
BBB	blood-brain barrier
BCA	bicinchoninic acid
BDNF	brain derived neurotrophic factor
BGH	bovine growth hormone
Bmax	dose causing maximal effect
BSA	bovine serum albumin
C	cysteine
CDR	compementarity determining region
CED	convection enhanced diffusion
CHO	Chinese hamster ovary
CMV	cytomegalovirus
CNTF	ciliary neurotrophic factor
DC	dilutional cloning

DHFR	dihydrofolate reductase
ECD	extracellular domain
ED50	effective dose causing 50% saturation
FR	framework region
FS	flanking sequence
FWD	forward
GDNF	glial derived neurotrophic factor
GFR	GDNF receptor
HC	heavy chain
HIR	human insulin receptor
HIRMAb	MAb to HIR
HIRMAb-GDNF	fusion protein of HIRMAb and GDNF
HPLC	high pressure liquid chromatography
HT	hypoxanthine-thymidine
ICV	intra-cerebroventricular
ID	injected dose
IgG	immunoglobulin G
LC	light chain
MAb	monoclonal antibody
MAH	mouse anti-human IgG
MCAO	middle cerebral artery occlusion
MTX	methotrexate
MW	molecular weight
N	asparagine
nt	nucleotide
ODN	oligodeoxynucleotide
pA	poly-adenylation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS plus Tween-20
PCR	polymerase chain reaction
PD	Parkinson's disease
pI	isoelectric point
RAG	rabbit anti-goat IgG
REV	reverse
RNase A	ribonuclease A
RT	reverse transcriptase
RT	room temperature

SDM	site-directed mutagenesis
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
Ser	serine
SFM	serum free medium
SMA	spinal muscular atrophy
TH	tyrosine hydroxylase
TTC	triphenyltetrazolium chloride
TV	tandem vector
UTV	universal TV
VH	variable region of heavy chain
VL	variable region of light chain

I. Introduction

[0054]   The blood brain barrier is a limiting factor in the delivery of many peripherally-administered agents to the central nervous system. The present invention addresses three factors that are important in delivering an agent across the BBB to the CNS: 1) a pharmacokinetic profile for the agent that allows sufficient time in the peripheral circulation for the agent to have enough contact with the BBB to traverse it; 2) modification of the agent to allow it to cross the BBB; and 3) retention of activity of the agent once across the BBB. Various aspects of the invention address these factors, by providing fusion structures (e.g., fusion proteins) of an agent (e.g., a therapeutic agent) covalently linked to a structure that causes the agent to have increased serum half life, to be transported across the BBB, and/or to retain some or all of its activity in the brain while still attached to the structure.

[0055]   Accordingly, in one aspect, the invention provides compositions and methods that utilize an agent covalently linked to a structure capable of crossing the blood brain barrier (BBB). The compositions and methods are useful in transporting agents, e.g. therapeutic agents such as neurotherapeutic agents, from the peripheral blood and across the BBB into the CNS. Neurotherapeutic agents useful in the invention include neurotrophins, e.g. brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF). In some embodiments, the structure that is capable of crossing the BBB is capable of binding to an endogenous BBB receptor mediated transport system and crossing the BBB. In some embodiments, the structure that is capable of crossing the BBB is an antibody, e.g., a monoclonal antibody (MAb) such as a chimeric MAb.

[0056]   In some embodiments, the invention provides a fusion protein that includes a structure capable of crossing the BBB covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain a proportion (e.g., 10-100%) of their activities or

their binding affinities for their respective receptors, compared to their activities or their binding affinities for their respective receptors as separate entities.

[0057]   In another aspect, the invention provides a composition containing a cationic therapeutic peptide covalently linked to an immunoglobulin, where the cationic therapeutic peptide in the composition has a serum and/or circulating half-life that is an average of at least about five fold greater than the serum and/or circulating half-life of the cationic therapeutic peptide alone.

[0058]   The invention also provides nucleic acids coding for peptides and proteins. In some embodiments, the invention provides a single nucleic acid sequence that contains a gene coding for a light chain of an immunoglobulin and a gene coding for a fusion protein made up of a heavy chain of the immunoglobulin covalently linked to a peptide. In some embodiments the peptide of the fusion protein is a therapeutic peptide, e.g., a neurotherapeutic peptide such as a neurotrophin. The invention also provides vectors containing the nucleic acids of the invention, and cells containing the vectors. Further provided are methods of manufacturing an immunoglobulin fusion protein, where the fusion protein contains an immunoglobulin heavy chain fused to a therapeutic agent, where the methods include integrating into a eukaryotic cell a single tandem expression vector in which both the immunoglobulin light chain gene and the gene for the immunoglobulin heavy chain fused to the therapeutic agent are incorporated into a single piece of DNA.

[0059]   The invention further provides therapeutic compositions, such as pharmaceutical compositions that contain an agent covalently linked to a structure capable of crossing the blood brain barrier (BBB) and a pharmaceutically acceptable excipient. In some embodiments, the invention provides a composition for treating a neurological disorder that includes a BDNF or a GDNF (e.g., human GDNF) covalently linked to an immunoglobulin that is capable of crossing the blood brain barrier, wherein the composition is capable of crossing the BBB in an amount that is effective in treating the neurological disorder.

[0060]   The invention also provides methods for treating a neurological disorder in an individual that include peripherally administering to the individual an effective amount of one or more of the compositions of the invention, optionally in combination with other therapy for the disorder.

II. Definitions

[0061]   As used herein, an "agent" includes any substance that is useful in producing an effect, including a physiological or biochemical effect in an organism. A "therapeutic agent" is a substance that produces or is intended to produce a therapeutic effect, i.e., an effect that leads to amelioration, prevention, and/or complete or partial cure of a disorder. A "therapeutic effect," as that term is used herein, also includes the production of a condition that is better than the average or normal condition in an individual that is not suffering from a disorder, i.e., a supranormal effect such as improved cognition, memory, mood, or other characteristic attributable at least in part to the functioning of the CNS, compared to the normal or average state. A "neurotherapeutic agent" is an agent that produces a therapeutic effect in the CNS. A "therapeutic peptide" includes therapeutic agents that consists of a peptide. A "cationic therapeutic peptide" encompasses therapeutic peptides

whose isoelectric point is above about 7.4, in some embodiments, above about 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, or above about 12.5. A subcategory of cationic therapeutic peptides is cationic neurotherapeutic peptides.

[0062]   As used herein, a "peptide that is active in the central nervous system (CNS)" includes peptides that have an effect when administered to the CNS. The effect may be a therapeutic effect or a non-therapeutic effect, e.g., a diagnostic effect or an effect useful in research. If the effect is a therapeutic effect, then the peptide is also a therapeutic peptide. A therapeutic peptide that is also a peptide that is active in the CNS is encompassed by the term "neurotherapeutic peptide," as used herein.

[0063]   "Treatment" or "treating" as used herein includes achieving a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder or condition being treated. For example, in an individual with a neurological disorder, therapeutic benefit includes partial or complete halting of the progression of the disorder, or partial or complete reversal of the disorder. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological or psychological symptoms associated with the underlying condition such that an improvement is observed in the patient, notwithstanding the fact that the patient may still be affected by the condition. A prophylactic benefit of treatment includes prevention of a condition, retarding the progress of a condition (e.g., slowing the progression of a neurological disorder), or decreasing the likelihood of occurrence of a condition. As used herein, "treating" or "treatment" includes prophylaxis.

[0064]   As used herein, the term "effective amount" can be an amount sufficient to effect beneficial or desired results, such as beneficial or desired clinical results, or enhanced cognition, memory, mood, or other desired CNS results. An effective amount is also an amount that produces a prophylactic effect, e.g., an amount that delays, reduces, or eliminates the appearance of a pathological or undesired condition. Such conditions of the CNS include dementia, neurodegenerative diseases as described herein, suboptimal memory or cognition, mood disorders, general CNS aging, or other undesirable conditions. An effective amount can be administered in one or more administrations. In terms of treatment, an "effective amount" of a composition of the invention is an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of a disorder, e.g., a neurological disorder. An "effective amount" may be of any of the compositions of the invention used alone or in conjunction with one or more agents used to treat a disease or disorder. An "effective amount" of a therapeutic agent within the meaning of the present invention will be determined by a patient's attending physician or veterinarian. Such amounts are readily ascertained by one of ordinary skill in the art and will a therapeutic effect when administered in accordance with the present invention. Factors which influence what a therapeutically effective amount will be include, the specific activity of the therapeutic agent being used, the type of disorder (e.g., acute vs. chronic neurological disorder), time elapsed since the initiation of the disorder, and the age, physical condition, existence of other disease states, and

nutritional status of the individual being treated. Additionally, other medication the patient may be receiving will affect the determination of the therapeutically effective amount of the therapeutic agent to administer.

[0065]   A "subject" or an "individual," as used herein, is an animal, for example, a mammal. In some embodiments a "subject" or an "individual" is a human. In some embodiments, the subject suffers from a neurological disorder.

[0066]   In some embodiments, an agent is "administered peripherally" or "peripherally administered." As used herein, these terms refer to any form of administration of an agent, e.g., a therapeutic agent, to an individual that is not direct administration to the CNS, i.e., that brings the agent in contact with the non-brain side of the blood-brain barrier. "Peripheral administration," as used herein, includes intravenous, subcutaneous, intramuscular, intraperitoneal, transdermal, inhalation, transbuccal, intranasal, rectal, and oral administration.

[0067]   A "pharmaceutically acceptable carrier" or "pharmaceutically acceptable excipient" herein refers to any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Such carriers are well known to those of ordinary skill in the art. A thorough discussion of pharmaceutically acceptable carriers/excipients can be found in *Remington's Pharmaceutical Sciences*, Gennaro, AR, ed., 20th edition, 2000: Williams and Wilkins PA, USA.. Exemplary pharmaceutically acceptable carriers can include salts, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. For example, compositions of the invention may be provided in liquid form, and formulated in saline based aqueous solution of varying pH (5-8), with or without detergents such polysorbate-80 at 0.01-1%, or carbohydrate additives, such mannitol, sorbitol, or trehalose. Commonly used buffers include histidine, acetate, phosphate, or citrate.

[0068]   A "recombinant host cell" or "host cell" refers to a cell that includes an exogenous polynucleotide, regardless of the method used for insertion, for example, direct uptake, transduction, f-mating, or other methods known in the art to create recombinant host cells. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

[0069]   The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. That is, a description directed to a polypeptide applies equally to a description of a peptide and a description of a protein, and vice versa. The terms apply to naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues is a non-naturally occurring amino acid, e.g., an amino acid analog. As used herein, the terms encompass amino acid chains of any length, including full length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds.

[0070]   The term "amino acid" refers to naturally occurring and non-naturally occurring amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to

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the naturally occurring amino acids. Naturally encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrolysine and selenocysteine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, such as, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (such as, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid.

[0071]   Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0072]   The term "nucleic acid" refers to deoxyribonucleotides, deoxyribonucleosides, ribonucleosides, or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless specifically limited otherwise, the term also refers to oligonucleotide analogs including PNA (peptidonucleic acid), analogs of DNA used in antisense technology (phosphorothioates, phosphoroamidates, and the like). Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof, including, but not limited to, degenerate codon substitutions, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al., Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al., J. Biol. Chem.* 260:2605-2608 (1985); and Cassol *et al.* (1992); Rossolini *et al., Mol. Cell. Probes* 8:91-98 (1994)).

[0073]   The terms "isolated" and "purified" refer to a material that is substantially or essentially removed from or concentrated in its natural environment. For example, an isolated nucleic acid may be one that is separated from the nucleic acids that normally flank it or other nucleic acids or components (proteins, lipids, etc...) in a sample. In another example, a polypeptide is purified if it is substantially removed from or concentrated in its natural environment. Methods for purification and isolation of nucleic acids and peptides are well known in the art.

III. The blood brain barrier

[0074]   In one aspect, the invention provides compositions and methods that utilize an agent covalently linked to a structure capable of crossing the blood brain barrier (BBB). The compositions and methods are useful in transporting agents, e.g. therapeutic agents such as

neurotherapeutic agents, from the peripheral blood and across the BBB into the CNS. As used herein, the "blood-brain barrier" refers to the barrier between the peripheral circulation and the brain and spinal cord which is formed by tight junctions within the brain capillary endothelial plasma membranes, creates an extremely tight barrier that restricts the transport of molecules into the brain, even molecules as small as urea, molecular weight of 60 Da. The blood-brain barrier within the brain, the blood-spinal cord barrier within the spinal cord, and the blood-retinal barrier within the retina, are contiguous capillary barriers within the central nervous system (CNS), and are collectively referred to as the blood-brain barrier or BBB.

[0075]   The BBB is a limiting step in the development of new neurotherapeutics, diagnostics, and research tools for the brain and CNS. In general, large molecule therapeutics such as recombinant proteins, antisense drugs, gene medicines, monoclonal antibodies, or RNA interference (RNAi)-based drugs, do not cross the BBB in pharmacologically significant amounts. While it is generally assumed that small molecule drugs can cross the BBB, in fact, <2% of all small molecule drugs are active in the brain owing to the lack transport across the BBB. A molecule must be lipid soluble and have a molecular weight less than 400 Daltons (Da) in order to cross the BBB in pharmacologically significant amounts, and the vast majority of small molecules do not have these dual molecular characteristics. Therefore, most potentially therapeutic, diagnostic, or research molecules do not cross the BBB in pharmacologically active amounts. So as to bypass the BBB, invasive transcranial drug delivery strategies are used, such as intracerebro-ventricular (ICV) infusion, intracerebral (IC) administration, and convection enhanced diffusion (CED). Transcranial drug delivery to the brain is expensive, invasive, and largely ineffective. The ICV route delivers BDNF only to the ependymal surface of the brain, not into brain parenchyma, which is typical for drugs given by the ICV route. The IC administration of a neurotrophin, such as nerve growth factor (NGF), only delivers drug to the local injection site, owing to the low efficiency of drug diffusion within the brain. The CED of neurotrophin results in preferential fluid flow through the white matter tracts of brain, which causes demyelination, and astrogliosis.

[0076]   The present invention offers an alternative to these highly invasive and generally unsatisfactory methods for bypassing the BBB, allowing agents, e.g., neuroprotective factors to cross the BBB from the peripheral blood. It is based on the use of endogenous transport systems present in the BBB to provide a mechanism to transport a desired substance from the peripheral blood to the CNS.

         A. Transport systems

[0077]   In some embodiments, the invention provides compositions that include a structure that binds to a BBB receptor mediated transport system, coupled to an agent for which transport across the BBB is desired, e.g., a neurotherapeutic agent. The compositions and methods of the invention may utilize any suitable structure that is capable of transport by the selected endogenous BBB receptor-mediated transport system, and that is also capable of attachment to the desired agent. In

some embodiments, the structure is an antibody. In some embodiment the antibody is a monoclonal antibody (MAb), e.g., a chimeric MAb.

[0078]   Endogenous BBB receptor-mediated transport systems The BBB has been shown to have specific receptors that allow the transport from the blood to the brain of several macromolecules; these transporters are suitable as transporters for compositions of the invention. Endogenous BBB receptor-mediated transport systems useful in the invention include those that transport insulin, transferrin, insulin-like growth factors 1 and 2 (IGF1 and IGF2), leptin, and lipoproteins. In some embodiments, the invention utilizes a structure that is capable of crossing the BBB via the endogenous insulin BBB receptor-mediated transport system, e.g., the human endogenous insulin BBB receptor-mediated transport system.

B. Structures that bind to a BBB receptor mediated transport system

[0079]   One noninvasive approach for the delivery of drugs to the CNS is to attach the agent of interest to a structure, e.g., molecule that binds with receptors on the BBB. The structure then serves as a vector for transport of the agent across the BBB. Such structures are referred to herein as "molecular Trojan horses (MTH)." Typically, though not necessarily, a MTH is an exogenous peptide or peptidomimetic moiety (e.g., a MAb) capable of binding to an endogenous BBB receptor mediated transport system that traverses the BBB on the endogenous BBB receptor-mediated transport system. In certain embodiments, the MTH can be an antibody to a receptor of the transport system, e.g., the insulin receptor. In some embodiments, the antibody is a monoclonal antibody (MAb). In some embodiments, the MAb is a chimeric MAb. Thus, despite the fact that Abs normally are excluded from the brain, they can be an effective vehicle for the delivery of molecules into the brain parenchyma if they have specificity for receptors on the BBB.

[0080]   Accordingly, antibodies are particularly useful in embodiments of the invention, especially MAbs. Certain receptor-specific MAbs may mimic the endogenous ligand and function as a MTH and traverse a plasma membrane barrier via transport on the specific receptor system. In certain embodiments, the MTH is a MAb to the human insulin receptor (HIR) on the human BBB. The HIR MAb binds an exofacial epitope on the human BBB HIR and this binding enables the MAb to traverse the BBB via a transport reaction that is mediated by the human BBB insulin receptor.

[0081]   An "antibody," as that term is used herein, includes reference to any molecule, whether naturally-occurring, artificially induced, or recombinant, which has specific immunoreactive activity. Generally, though not necessarily, an antibody is a protein that includes two molecules, each molecule having two different polypeptides, the shorter of which functions as the light chains of the antibody and the longer of which polypeptides function as the heavy chains of the antibody. Normally, as used herein, an antibody will include at least one variable region from a heavy or light chain. Additionally, the antibody may comprise combinations of variable regions. The combination may include more than one variable region of a light chain or of a heavy chain. The antibody may also include variable regions from one or more light chains in combination with variable regions of one or more heavy chains. An antibody can be an immunoglobulin molecule obtained by in vitro or

in vivo generation of the humoral response, and includes both polyclonal and monoclonal antibodies. Furthermore, the present invention includes antigen binding fragments of the antibodies described herein, such as Fab, Fab', F(ab)$_2$, and Fv fragments, fragments comprised of one or more CDRs, single-chain antibodies (e.g., single chain Fv fragments (scFv)), disulfide stabilized (dsFv) Fv fragments, heteroconjugate antibodies (e.g., bispecific antibodies), pFv fragments, heavy chain monomers or dimers, light chain monomers or dimers, and dimers consisting of one heavy chain and one light chain. Such antibody fragments may be produced by chemical methods, e.g., by cleaving an intact antibody with a protease, such as pepsin or papain, or via recombinant DNA techniques, e.g., by using host cells transformed with truncated heavy and/or light chain genes. Synthetic methods of generating such fragments are also contemplated. Heavy and light chain monomers may similarly be produced by treating an intact antibody with a reducing agent, such as dithiothreitol or beta-mercaptoethanol, or by using host cells transformed with DNA encoding either the desired heavy chain or light chain or both. An antibody immunologically reactive with a particular antigen can be generated in vivo or by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors.

[0082]   A "chimeric" antibody includes an antibody derived from a combination of different mammals. The mammal may be, for example, a rabbit, a mouse, a rat, a goat, or a human. The combination of different mammals includes combinations of fragments from human and mouse sources.

[0083]   In some embodiments, an antibody of the present invention is a monoclonal antibody (MAb), typically a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas.

[0084]   For use in humans, a chimeric MAb is preferred that contains enough human sequence that it is not significantly immunogenic when administered to humans, e.g., at least about 80% human and about 20% mouse, or about 85% human and about 15% mouse, or about 90% human and about 10% mouse, or about 95% human and 5% mouse, or greater than about 95% human and less than about 5% mouse. Chimeric antibodies to the human BBB insulin receptor with sufficient human sequences for use in the invention are described in, e.g., Coloma et al. (2000) *Pharm. Res.* 17: 266-274, which is incorporated by reference herein in its entirety. A more highly humanized form of the HIR MAb can also be engineered, and the humanized HIRMAb has activity comparable to the murine HIRMAb and can be used in embodiments of the invention. See, e.g., U.S. Patent Application Publication No. 20040101904, filed 11/27/02, incorporated by reference herein in its entirety.

[0085]   Antibodies used in the invention may be glycosylated or non-glycosylated. If the antibody
is glycosylated, any pattern of glycosylation that does not significantly affect the function of the
antibody may be used. Glycosylation can occur in the pattern typical of the cell in which the
antibody is made, and may vary from cell type to cell type. For example, the glycosylation pattern
of a monoclonal antibody produced by a mouse myeloma cell can be different than the
glycosylation pattern of a monoclonal antibody produced by a transfected Chinese hamster ovary
(CHO) cell. In some embodiments, the antibody is glycosylated in the pattern produced by a
transfected Chinese hamster ovary (CHO) cell.

[0086]   Accordingly, in some embodiments, a genetically engineered HIR MAb, with the desired
level of human sequences, is fused to an agent for which transport across the BBB is desired, e.g., a
neurotherapeutic agent such as a neurotrophin such as human BDNF, to produce a recombinant
fusion protein that is a bi-functional molecule. The recombinant therapeutic neuroprotective
factor/HIRMAb is able to both (i) cross the human BBB, via transport on the BBB HIR, and (ii)
activate the factor's target, e.g., neuronal BDNF receptor, trkB, to cause neurotherapeutic effects
once inside the brain, following peripheral administration.

IV. Agents for Transport Across the BBB

[0087]   The agent for which transport across the BBB is desired may be any suitable substance for
introduction into the CNS. Generally, the agent is a substance for which transport across the BBB
is desired, which does not, in its native form, cross the BBB in significant amounts. The agent may
be, e.g., a therapeutic agent, a diagnostic agent, or a research agent. Diagnostic agents include
peptide radiopharmaceuticals, such as radiolabeled epidermal growth factor (EGF) for imaging
brain cancer (Kurihara and Pardridge (1999) *Canc. Res.* 54: 6159-6163), and amyloid peptides for
imaging brain amyloid such as in Alzheimers disease (Lee et al (2002) *J. Cereb. Blood Flow
Metabol.* 22: 223-231). In some embodiments, the agent is a therapeutic agent, such as a
neurotherapeutic agent. Apart from neurotrophins, potentially useful therapeutic protein agents
include recombinant enzymes for lysosomal storage disorders (see, e.g., U.S. Patent Application
Publication No. 20050142141, filed 2/17/05, incorporated by reference herein in its entirety),
monoclonal antibodies that either mimic an endogenous peptide or block the action of an
endogenous peptide, polypeptides for brain disorders, such as secretin for autism (Ratliff-Schaub et
al (2005) Autism 9: 256-265), opioid peptides for drug or alchol addiction (Cowen et al, (2004) J.
Neurochem. 89: 273-285), or neuropeptides for appetite control (Jethwa et al (2005) Am. J. Physiol.
289: E301-305). In some embodiments, the agent is a neurotrophic factor, also referred to herein as
a "neurotrophin." Thus, in some embodiments, the invention provides compositions and methods
that utilize a neurotrophin. In some embodiments, a single neurotrophin may be used. In others,
combinations of neurotrophins are used. In some embodiments, the invention utilizes a brain-
derived neurotrophic factor (BDNF). In other embodiments, the invention utilizes a glial-derived
neurotrophic factor.

A. Neurotrophins

[0088]   Many neurotrophic factors are neuroprotective in brain, but do not cross the blood-brain barrier. These factors are suitable for use in the compositions and methods of the invention and include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-4/5, fibroblast growth factor (FGF)-2 and other FGFs, neurotrophin (NT)-3, erythropoietin (EPO), hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF)-α, TGF-β, vascular endothelial growth factor (VEGF), interleukin-1 receptor antagonist (IL-1ra), ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), neurturin, platelet-derived growth factor (PDGF), heregulin, neuregulin, artemin, persephin, interleukins, granulocyte-colony stimulating factor (CSF), granulocyte-macrophage-CSF, netrins, cardiotrophin-1, hedgehogs, leukemia inhibitory factor (LIF), midkine, pleiotrophin, bone morphogenetic proteins (BMPs), netrins, saposins, semaphorins, and stem cell factor (SCF). Particularly useful in some embodiments of the invention utilizing neurotrophins that are used as precursors for fusion proteins that cross the BBB are those that naturally form dimeric structures, similar to BDNF. Certain neurotrophins such as BDNF or NT-3 may form hetero-dimeric structures, and in some embodiments the invention provides a fusion protein constructed of one neurotrophin monomer fused to one chain (e.g., a light or heavy chain) of an antibody, e.g., of the HIRMAb, and another neurotrophin monomer fused to the second chain (e.g., a light or heavy chain) of the antibody. Typically, the molecular weight range of recombinant proteins that may be fused to the molecular Trojan horse ranges from 1000 Daltons to 500,000 Daltons.

B. Brain-Derived Neurotrophic Factor

[0089]   One particularly useful neurotrophin in embodiments of the invention is brain-derived neurotrophic factor (BDNF). In experimental models of chronic neurodegenerative disease such as prion diseases, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), or amyotrophic lateral sclerosis (ALS), the direct intracerebral injection of BDNF is neuroprotective.

[0090]   In studies demonstrating the pharmacologic efficacy of BDNF in experimental brain disease, it is necessary to administer the neurotrophin directly into the brain following a transcranial drug delivery procedure. The transcranial drug delivery is required because BDNF does not cross the brain capillary wall, which forms the blood-brain barrier (BBB) in vivo. Owing to the lack of transport of BDNF across the BBB, it is not possible for the neurotrophin to enter the CNS, including the brain or spinal cord, following a peripheral administration unless the BBB is experimentally disrupted. Clinical trials showed that subcutaneous administration of BDNF was not effective in the treatment of chronic neurodegenerative conditions, which derives from the lack of transport of BDNF across the BBB. The lack of utility of BDNF as a CNS therapeutic following peripheral administration is expected and follows from the limiting role that is played by the BBB in the development of neurotherapeutics, especially large molecule drugs such as BDNF. BDNF

does not cross the BBB, and the lack of transport of the neurotrophin across the BBB prevents the molecule from being pharmacologically active in the brain following peripheral administration. The lack of BDNF transport across the BBB means that the neurotrophin must be directly injected into the brain across the skull bone to be pharmacologically active in the CNS. However, when the BDNF is fused to a Trojan horse such as the HIR MAb, this neurotrophin is now able to enter brain from blood following a non-invasive peripheral route of administration such as intravenous intramuscular, subcutaneous, intraperitoneal, or even oral administration. Owing to the BBB transport properties of this new class of molecule, it is not necessary to administer the BDNF directly into the CNS with an invasive delivery procedure requiring penetration of the skull or spinal canal. The reformulated fusion protein of the BDNF variant and the HIR MAb now enables entry of BDNF into the brain from the blood, and the development of BDNF as a neurotherapeutic for human diseases.

[0091]　　　The forms of BDNF used in various embodiments of the invention may include pharmaceutically acceptable salts and prodrugs, and prodrugs of the salts, polymorphs, hydrates, solvates, biologically-active fragments, biologically active variants and stereoisomers of the naturally-occurring BDNF, as well as agonist, mimetic, and antagonist variants of the naturally-occurring BDNF and polypeptide fusions thereof. Variants that include one or more deletions, substitutions, or insertions in the natural sequence of the BDNF, in particular truncated versions of the native BDNF comprising deletion of one or more amino acids at the amino terminus, carboxyl terminus, or both, may also be used in certain embodiments.

[0092]　　　In some embodiments, the invention utilizes a carboxy-truncated variant of the native BDNF, e.g., a variant in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 amino acids are absent from the carboxy-terminus of the BDNF. BDNF variants include the complete 119 amino acid BDNF, the 117 or 118 amino acid variant with a truncated carboxyl terminus, variants with a truncated amino terminus, or variants with up to about a 20, 30, or 40% change in amino acid composition, as long as the fusion protein variant still binds to the brain neuroprotection receptor with high affinity. When an Ab, e.g., a MAb such as HIRMAb is used, additional fusion protein variants can be produced with the substitution of amino acids within either the framework region (FR) or the complementarity determining region (CDR) of either the light chain or the heavy chain of the Ab, e.g., HIRMAb, as long as the fusion protein binds with high affinity to the endogenous receptor, e.g., HIR to promote transport across the human BBB. Additional fusion protein variants can be produced by changing the composition or length of the linker peptide separating the fusion protein from the HIRMAb.

[0093]　　　In some embodiments, the full-length 119 a.a. sequence of BDNF is utilized (SEQ ID NO: 39). In some embodiments, a one amino-acid carboxy-truncated variant of BDNF is utilized (amino acids 1-118 of SEQ ID NO: 39). In some embodiments, a two amino-acid carboxy-truncated variant of BDNF is utilized (amino acids 1-117 of SEQ ID NO: 39). In some embodiments, a three amino-acid carboxy-truncated variant of BDNF is utilized (amino acids 1-116 of SEQ ID NO: 39).

In some embodiments, a four amino-acid carboxy-truncated variant of BDNF is utilized (amino acids 1-115 of SEQ ID NO: 39). In some embodiments, a five amino-acid carboxy-truncated variant of BDNF is utilized (amino acids 1-114 of SEQ ID NO: 39).

[0094]   The sequence of human BDNF is given in SEQ ID NO: 39. In some embodiments, the invention utilizes a BDNF that is about 60, 70, 80, 90, 95, 99, or 100% identical with the sequence of SEQ ID NO: 39, or a truncated version thereof, e.g., the 117 or 118 amino acid variant with a one- or two-amino acid truncated carboxyl terminus, or variants with a truncated amino terminus. In some embodiments, the invention utilizes a two amino-acid carboxy-truncated 117 amino acid variant human BDNF with a sequence that is at least about 60, 70, 80, 90, 95, 99 or 100% identical to the sequence of amino acids 466-582 of SEQ ID NO: 24. In some embodiments, the invention utilizes a two amino-acid carboxy-truncated human 117 amino acid BDNF with a sequence that includes amino acids 466-582 of SEQ ID NO: 24.

[0095]   Accordingly, BDNFs useful in the invention include peptides having at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or greater than 95% or greater than 99% sequence identity, e.g., 100% sequence identity, to the amino acid sequences disclosed herein.

C. Glial-Derived Neurotrophic Factor.

[0096]   Another particularly useful neurotrophin in embodiments of the invention is glial-derived neurotrophic factor (GDNF). GDNF is a neurotrophic factor that can be used in the treatment of many acute and chronic brain diseases. However, the lack of transport of GDNF across the BBB has prevented the development of this molecule as a neurotherapeutic for the brain and spinal cord.

[0097]   Acute brain conditions that can be treated by the GDNF-containing compositions described herein include, but are not limited to, spinal cord injury, brain injury (e.g., traumatic brain injury), focal brain ischemia, and global brain ischemia.

[0098]   Chronic brain conditions that can be treated by the GDNF-containing compositions described herein include, but are not limited to neurodegenerative disease such as Parkinson's disease, motor neuron disease (e.g., spinal cord motor neuron disease such as amyotrophic lateral sclerosis); and substance abuse, e.g., addiction, including drug addiction and alchohol addiction.

[0099]   The forms of GDNF used in various embodiments of the invention may include acceptable salts and prodrugs, and prodrugs of the salts, polymorphs, hydrates, solvates, biologically-active fragments, biologically active variants and stereoisomers of the naturally-occurring GDNF (e.g., human GDNF and mature human GDNF), as well as agonist, mimetic, and antagonist variants of the naturally-occurring GDNF and polypeptide fusions thereof. Variants that include one or more deletions, substitutions, or insertions in the natural sequence of the GDNF may also be used in certain embodiments. insofar as the variants retain binding and/or activation of GFRα1. The structure-function relationship of GDNF and its ability to bind and activate GFRα1 has been studied extensively. See, e.g., Eketjäl *et al* (1999), *EMBO J*, 18(21):5901-5910; and Baloh *et al* (2000), *J Biol Chem*, 275(5):3412-3420. For example mutations known to be particularly sensitive to

mutation include, e.g., the following residues in rat GDNF: D52, E61, E62, D116, I64, L114, L118, Y120, I122, and C101. Functional assays for GDNF are known in the art. See, e.g., Eketjäl *et al*, or Baloh *et al supra*. Functional assays for GDNF are also described in Example 15 herein.

[00100]   When an Ab, e.g., a MAb such as HIRMAb is used, additional fusion protein variants can be produced with the substitution of amino acids within either the framework region (FR) or the complementarity determining region (CDR) of either the light chain or the heavy chain of the Ab, e.g., HIRMAb, as long as the fusion protein binds with high affinity to the endogenous receptor, e.g., HIR to promote transport across the human BBB. Additional fusion protein variants can be produced by changing the composition or length of the linker peptide separating the fusion protein from the HIRMAb.

[00101]   In some embodiments, the full-length 211 a.a. sequence of human prepro GDNF is utilized (GenBank P39905). The sequence of human preproGDNF is given in SEQ ID NO: 44.   In other embodiments, the mature form of human GDNF comprising amino acids Ser-78 to Ile 211 (SEQ ID NO: 52) is utilized:

SPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYET KEEL

IFRYCSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLSFLDDNLVYHILRK HSAKRCGCI (SEQ ID NO:52).

[00102]   In some embodiments, the invention utilizes a GDNF that is about 60, 70, 80, 90, 95, 99, or 100% identical with the sequence of SEQ ID NO:52.

[00103]   Accordingly, GDNFs useful in the invention include peptides having at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or greater than 95% or greater than 99% sequence identity, e.g., 100% sequence identity, to the amino acid sequences disclosed herein

[00104]   Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., *Bull. Math. Bio.* 48:603 (1986), and Henikoff and Henikoff, *Proc. Natl. Acad. Sci.* USA 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff and Henikoff (ibid.). The percent identity is then calculated as: ([Total number of identical matches]/[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences])(100).

[00105]   Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of another peptide. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci.* USA 85:2444 (1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (e.g., SEQ ID NO:44 or SEQ ID NO:52) and a

test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, SIAM J. *Appl. Math.* 26:787 (1974)), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

[00106]   The present invention also includes peptides having a conservative amino acid change, compared with an amino acid sequence disclosed herein. Many such changes have been described specifically. More generally, for example, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NO:52. In some embodiments sequence variants include conservative amino acid substitutions, e.g., an alkyl amino acid is substituted for an alkyl amino acid in a GDNF peptide amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in a GDNF peptide amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in a GDNF peptide amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in a GDNF peptide amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in a GDNF peptide amino acid sequence, a basic amino acid is substituted for a basic amino acid in GDNF peptide amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in a GDNF peptide amino acid sequence. Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine. The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci.* USA 89:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed above), the language "conservative amino acid

substitution" preferably refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

[00107]   It also will be understood that amino acid sequences may include additional residues, such as additional N- or C-terminal amino acids, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence retains sufficient biological protein activity to be functional in the compositions and methods of the invention.

[00108]   In some embodiments, where GDNF sequence variants (e.g., variants of SEQ ID NO:52) are to be utilized, mutation tolerance prediction programs can be used to greatly reduce the number of non-functional sequence variants that would be generated by strictly random mutagenesis. Various programs for predicting the effects of amino acid substitutions in a protein sequence on protein function (e.g., SIFT, PolyPhen, PANTHER PSEC, PMUT, and TopoSNP) are described in, e.g., Henikoff *et al.*, (2006), *Annu. Rev. Genomics Hum. Genet.*, 7:61-80..

V. Compositions

[00109]   Compositions of the invention are useful in one or more of: increasing serum half-life of a cationic compound, transporting an agent across the BBB, and/or retaining activity of the agent once transported across the BBB. Accordingly, in some embodiments, the invention provides compositions containing a neurotherapeutic agent covalently linked to a structure that is capable of crossing the blood brain barrier (BBB), where the composition is capable of producing an average elevation of concentration in the brain of the neurotherapeutic agent of at least about 1, 2, 3, 4, 5, 10, 20, 30, 40, or 50 ng/gram brain following peripheral administration. The invention also provides compositions containing an agent that is covalently linked to a chimeric MAb to the human BBB insulin receptor. The invention further provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain an average of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99, or 100% of their activities, compared to their activities as separate entities. In certain embodiments, the invention further provides compositions that increase the serum half-life of cationic substances. The invention also provides pharmaceutical compositions that contain one or more compositions of the invention and a pharmaceutically acceptable excipient.

[00110]   In some embodiments, the invention provides compositions containing a neurotherapeutic agent covalently linked to a structure that is capable of crossing the blood brain barrier (BBB), where the composition is capable of producing an average elevation of concentration in the brain of

the neurotherapeutic agent of at least about 1, 2, 3, 4, 5, 10, 20, 30, 40, or 50 ng/gram brain following peripheral administration.

[00111]   "Elevation" of the agent is an increase in the brain concentration of the agent compared to the concentration of the agent administered alone (i.e., not covalently linked to a structure that is capable of crossing the BBB). In the case of agents for which only a small amount of the agent alone normally crosses the BBB, "elevation" may be an increase in the agent compared to resting brain levels. "Average" refers to the mean of at least three, four, five, or more than five measurements, preferably in different individuals. The individual in which the elevation is measured is a mammal, such as a rat, or, preferably, a primate, e.g., a monkey. An example of measurements of elevation of the level of a neurotherapeutic agent (BDNF) is given in Example 7.

[00112]   In some embodiments, the structure that is capable of crossing the BBB utilizes an endogenous BBB receptor mediated transport system, such as a system that utilizes the insulin receptor, transferrin receptor, leptin receptor, LDL receptor, or IGF receptor. In some embodiments, the endogenous BBB receptor mediated transport system is the insulin BBB receptor mediated transport system. In some embodiments, the structure that is capable of crossing the BBB is an antibody, e.g., a monoclonal antibody (MAb) such as a chimeric MAb. The antibody can be a chimeric antibody with sufficient human sequence that it is suitable for administration to a human. The antibody can be glycosylated or nonglycosylated; in some embodiments, the antibody is glycosylated, e.g., in a glycosylation pattern produced by its synthesis in a CHO cell. In embodiments in which the structure is an antibody, the covalent linkage between the antibody and the neurotherapeutic agent may be a linkage between any suitable portion of the antibody and the neurotherapeutic agent, as long as it allows the antibody-agent fusion to cross the blood brain barrier and the neurotherapeutic agent to retain a therapeutically useful portion of its activity within the CNS. In certain embodiments, the covalent link is between one or more light chains of the antibody and the neurotherapeutic agent. In the case of a peptide neurotherapeutic agent (e.g., a neurotrophin such as GDNF), the peptide can be covalently linked by its carboxy or amino terminus to the carboxy or amino terminus of the light chain (LC) or heavy chain (HC) of the antibody. Any suitable linkage may be used, e.g., carboxy terminus of light chain to amino terminus of peptide, carboxy terminus of heavy chain to amino terminus of peptide, amino terminus of light chain to amino terminus of peptide, amino terminus of heavy chain to amino terminus of peptide, carboxy terminus of light chain to carboxy terminus of peptide, carboxy terminus of heavy chain to carboxy terminus of peptide, amino terminus of light chain to carboxy terminus of peptide, or amino terminus of heavy chain to carboxy terminus of peptide. In some embodiments, the linkage is from the carboxy terminus of the HC to the amino terminus of the peptide. It will be appreciated that a linkage between terminal amino acids is not required, and any linkage which meets the requirements of the invention may be used; such linkages between non-terminal amino acids of peptides are readily accomplished by those of skill in the art.

[00113]   In some embodiments, the invention utilizes BDNF, either the native form or truncated
variants. Strikingly, it has been found that fusion proteins of these forms of BDNF retain full
transport and activity. This is surprising because the neurotrophin is translated in vivo in cells as a
prepro form and the prepro-BDNF is then converted into mature BDNF following cleavage of the
prepro peptide from the amino terminus of the BDNF. In order to preserve the prepro form of the
BDNF, and the subsequent cleavability of the prepro peptide, it would seem to be necessary to fuse
the prepro BDNF to the amino terminus of either the HC or the LC of the targeting MAb. This
could, however, inhibit the binding of the MAb for the target antigen, since the complementarity
determining regions (CDR) of the heavy chain or light chain of the MAb molecule, which comprise
the antigen binding site of the MAb, are situated near the amino terminus of the heavy chain or light
chains of the antibody. Therefore, fusion of the prepro-neurotrophin to the amino terminus of the
antibody chains is expected to result in not only impairment of antibody activity, but also an
impairment of antibody folding following translation. The present invention shows the unexpected
finding that it is possible to fuse the mature form of a neurotrophin, such as a BDNF variant
(vBDNF), to the carboxyl terminus of the heavy chain of the HIR MAb. The production of this
new genetically engineered fusion protein creates a bi-functional molecule that binds with high
affinity to both the HIR and the trkB receptors.

[00114]   In other embodiments, the invention utilizes GDNF (e.g., mature human GDNF) or a
sequence variant of GDNF as described herein..

[00115]   In some embodiments, more than one molecule of the same neurotherapeutic agent is
attached to the structure that crosses the BBB. For example, in compositions of the invention where
a single neurotrophin is attached to an antibody, one molecule of the neurotrophin is attached to
each heavy chain, naturally producing a structure that is ideal for homodimer formation. This is the
case for compositions containing BDNF or GDNF. Neurotrophins such as BDNF or GDNF require
an obligatory formation of a homo-dimeric structure to be biologically active, and to bind with high
affinity to the cognate receptor, e.g. TrkB or GFRα1. A naturally occurring homo-dimeric structure
between two BDNF molecules is formed when the neurotrophin is fused to a carboxyl terminus of
the CH3 region of an IgG molecule, as illustrated in Figure 18. Without being bound by theory, it
is thought that this may account for the unexpected finding of essentially 100% of activity for the
BDNF when bound to the IgG (see, e.g., Fig. 24).

[00116]   In some embodiments, more than one type of neurotherapeutic agent can be attached to the
structure that is capable of crossing the blood brain barrier.   In some embodiments, 2, 3, 4, 5, 6, 7,
8, 9, 10, or more than 10 different neurotherapeutic agents may be attached to the structure that is
capable of crossing the blood brain barrier. In certain embodiments, 2 different neurotrophins are
attached to an antibody to an endogenous BBB receptor-mediated transport system. Any
combination of neurotrophins may be used. Particularly useful in some embodiments of the
invention are neurotrophins used as precursors for fusion proteins that cross the BBB are those that
naturally form dimeric structures, similar to BDNF or GDNF. Certain neurotrophins such as BDNF

or NT-3 may form hetero-dimeric structures, and in some embodiments the invention provides a fusion protein constructed of one neurotrophin monomer fused to one chain (e.g., heavy chain) of an antibody, e.g., of the HIRMAb, and another neurotrophin monomer fused to the second chain of the antibody. Typically, the molecular weight range of recombinant proteins that may be fused to the molecular Trojan horse ranges from 1000 Daltons to 500,000 Daltons.

[00117]   In some embodiments, more than one type of structure capable of crossing the BBB, e.g., molecular Trojan horse, may be used. The different structures may be covalently attached to a single neurotherapeutic agent, e.g., a single neurotrophin such as GDNF, or multiple neurotherapeutics, e.g., multiple neurotrophins, or any combination thereof. Thus, for example, in some embodiments either with the same neurotrophin attached to each MTH or a different neurotrophin attached, or combinations of neurotrophins attached. Thus the neuroprotective recombinant protein can be fused to multiple molecular Trojan horses that undergo receptor-mediated transport across the blood-brain barrier, including monoclonal antibodies to the insulin receptor, transferrin receptor, insulin-like growth factor (IGF) receptor, or the low density lipoprotein (LDL) receptor or the endogenous ligand, including insulin, transferrin, the IGFs, or LDL. Ligands that traverse the blood-brain barrier via absorptive-mediated transport may also be used as molecular Trojan horses including cationic proteins, or carbohydrate bearing proteins that bind to membrane lectins. The molecular weight range of molecular Trojan horses is 1000 Daltons to 500,000 Daltons.

[00118]   The covalent linkage between the structure capable of crossing the BBB and the neurotherapeutic agent may be direct, e.g., a peptide bond between the terminal amino acid of one peptide and the terminal amino acid of the other peptide to which it is linked, or indirect, via a linker. If a linker is used, it may be any suitable linker, e.g., a peptide linker. If a peptide linker is used, it may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 amino acids in length. In some embodiments, a three amino acid linker is used. In some embodiments, the linker has the sequence ser-ser-met. The covalent linkage may be cleavable, however this is not a requirement for activity of the system in some embodiments; indeed, an advantage of these embodiments of the present invention is that the fusion protein, without cleavage, is partially or fully active both for transport and for activity once across the BBB.

[00119]   In some embodiments, a noncovalent attachment may be used. An example of noncovalent attachment of the MTH, e.g., MAb, to the large molecule therapeutic neuroprotective factor is avidin/streptavidin-biotin attachment. Such an approach is further described in U.S. Patent Application No. 10/858,729, entitled "Anti-growth factor receptor avidin fusion proteins as universal vectors for drug delivery," filed April 21, 2005, which is hereby incorporated by reference in its entirety.

[00120]   The neurotherapeutic agent may be any suitable neurotherapeutic agent, such as a neurotrophin. In some embodiments, the neurotherapeutic agent is a neurotrophin such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-4/5, fibroblast

growth factor (FGF)-2 and other FGFs, neurotrophin (NT)-3, erythropoietin (EPO), hepatocyte
growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF)-α, TGF-β,
vascular endothelial growth factor (VEGF), interleukin-1 receptor antagonist (IL-1ra), ciliary
neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), neurturin, platelet-derived
growth factor (PDGF), heregulin, neuregulin, artemin, persephin, interleukins, granulocyte-colony
stimulating factor (CSF), granulocyte-macrophage-CSF, netrins, cardiotrophin-1, hedgehogs,
leukemia inhibitory factor (LIF), midkine, pleiotrophin, bone morphogenetic proteins (BMPs),
netrins, saposins, semaphorins, or stem cell factor (SCF). In some embodiments, the neurotrophin
is BDNF. The BDNF may be native BDNF or a variant BDNF. Some embodiments utilize a two
amino acid carboxyl-truncated variant. The BDNF can be a human BDNF. In some embodiments,
the BDNF contains a sequence that is about 60, 70, 80, 90, 95, 99, or 100% identical to the
sequence of amino acids 466-582 of SEQ ID NO: 24.

[00121]   In some embodiments, the invention provides compositions containing a neurotherapeutic
agent covalently linked to a structure that is capable of crossing the BBB where the composition is
capable of producing an average elevation of concentration in the brain of the neurotherapeutic
agent of at least about 1, 2, 3, 4, 5, 10, 20, 30, 40, or 50 ng/gram brain following peripheral
administration, where the neurotherapeutic agent is a neurotrophin and the structure that is capable
of crossing the BBB is a MAb to an endogenous BBB receptor mediated transport system. The
antibody can be glycosylated or nonglycosylated; in some embodiments, the antibody is
glycosylated, e.g., in a glycosylation pattern produced by its synthesis in a CHO cell. In certain
embodiments, the neurotrophin is GDNF, e.g., a mature human GDNF (SEQ ID NO:52) or a
sequence variant thereof. The MAb can be an antibody to the insulin BBB receptor mediated
transport system, e.g., a chimeric MAb. The antibody can be a chimeric antibody with sufficient
human sequence that it is suitable for administration to a human, e.g., at least about 80% human
sequence, e.g., 85%, 90%, 95%, or another percent human amino acid sequence from about 80% to
about 100% human sequence. In some embodiments, the insulin receptor is a human insulin
receptor and the GDNF is a human GDNF. In some embodiments, the GDNF contains a sequence
that is about 60, 70, 80, 90, 95, 99, or 100% identical to the sequence of SEQ ID NO: 44. The
GDNF can be covalently linked at its amino terminus to the carboxy terminus of the heavy chain of
the MAb, optionally with a linker between the termini, such as the three amino-acid linker ser-ser-
met, or the two amino acid linker ser-ser. In some embodiments, the heavy chain of the MAb
contains a sequence that is about 60, 70, 80, 90, 95, 99, or 100% identical to amino acids 20-462 of
SEQ ID NO: 24. In some embodiments, the light chain of the MAb contains a sequence that is
about 60, 70, 80, 90, 95, 99, or 100% identical to amino acids 21-234 of SEQ ID NO: 36.

[00122]   The invention also provides compositions containing an agent that is covalently linked to a
chimeric MAb to the human BBB insulin receptor. In some embodiments, the heavy chain of the
MAb is covalently linked to the agent to form a fusion protein. The agent can be any agent
described herein, i.e., any agent for which transport across the BBB is desired. In some

embodiments, the agent is a therapeutic agent, such as a neurotherapeutic agent as described herein, e.g., a neurotrophin such as GDNF

[00123]   Strikingly, it has been found that multifunctional fusion proteins of the invention, e.g., difunctional fusion proteins, retain a high proportion of the activity of the separate portions, e.g., the portion that is capable of crossing the BBB and the portion that is active in the CNS. Accordingly, the invention further provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the BBB and the peptide that is active in the central nervous system each retain an average of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99, or 100% of their activities, compared to their activities as separate entities. In some embodiments, the structure capapble of crossing the BBB, and the peptide that is active in the central nervous system each retain about 20% to about 80% of their activities (e.g., about 30% to about 70, or about 40% to about 60%) compared to their activities as separate entities. In some embodiments, the invention provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain an average of at least about 50% of their activities, compared to their activities as separate entities. In some embodiments, the invention provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain an average of at least about 60% of their activities, compared to their activities as separate entities. In some embodiments, the invention provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain an average of at least about 70% of their activities, compared to their activities as separate entities. In some embodiments, the invention provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain an average of at least about 80% of their activities, compared to their activities as separate entities. In some embodiments, the invention provides a fusion protein containing a structure capable of crossing the BBB, covalently linked to a peptide that is active in the central nervous system (CNS), where the structure capable of crossing the blood brain barrier and the peptide that is active in the central nervous system each retain an average of at least about 90% of their activities, compared to their activities as separate entities. In some embodiments, the structure capable of crossing the blood brain barrier retains at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99, or 100% of its activity, compared to its activity as a separate entity, and the peptide that is active in the central nervous

system retains at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99, or 100% of its activity, compared to its activity as a separate entity.

[00124]   As used herein, "activity" includes physiological activity, e.g., ability to cross the BBB and/or therapeutic activity and also binding affinity of the structures for their respective receptors.

[00125]   Transport of the structure capable of crossing the BBB across the BBB may be compared for the structure alone and for the structure as part of a fusion structure of the invention by standard methods. For example, pharmacokinetics and brain uptake of the fusion structure, e.g., fusion protein, by a model animal, e.g., a mammal such as a primate, may be used. Such techniques are illustrated in Example 7, which demonstrates pharmacokinetics and brain uptake of a fusion protein of the invention by the adult Rhesus monkey. Similarly, standard models for the function of an agent, e.g. the therapeutic or protective function of a therapeutic agent, may also be used to compare the function of the agent alone and the function of the agent as part of a fusion structure of the invention. See, e.g., Example 5, which demonstrates the activity of a neurotrophin alone and the same neurotrophin bound to a fusion protein in a model system (hypoxia-reoxygenation in human neural cells). In both Example 5 and Example 7, the fusion protein of the invention retained about 100% of the transport ability and the therapeutic function of its individual components, i.e., a structure capable of crossing the BBB (a MAb to the human insulin receptor) and a therapeutic agent (BDNF).

[00126]   Alternatively, binding affinity for receptors may be used as a marker of activity. Binding affinity for the receptor is compared for the structure alone and for the structure when part of the fusion protein. A suitable type of binding affinity assay is the competitive ligand binding assay (CLBA). For example, for fusion proteins containing MAbs to endogenous BBB receptor-mediated transport systems fused to a neurotrophin, a CLBA may be used both to assay the affinity of the MAb for its receptor and the neurotrophin for its receptor, either as part of the fusion protein or as separate entities, and percentage affinity calculated. If, as in some embodiments, the peptide that is active in the CNS is highly ionic, e.g., cationic, causing a high degree of non-specific binding, suitable measures should be taken to eliminate the nonspecific binding. See, e.g., Example 4. "Average" measurements are the average of at least three separate measurements.

[00127]   In embodiments of the above fusion proteins, the structure capable of crossing the blood brain barrier crosses the BBB on an endogenous BBB receptor-mediated transporter, such as a transporter selected from the group consisting of the insulin transporter, the transferrin transporter, the leptin transporter, the LDL transporter, and the IGF receptor. In some embodiments, the endogenous BBB receptor-mediated transporter is selected from the group consisting of the insulin transporter and the transferrin transporter. In some embodiments, the endogenous BBB receptor-mediated transporter is the insulin transporter, e.g., the human insulin transporter. The structure capable of crossing the BBB can be an antibody, e.g., a MAb such as a chimeric MAb. The antibody can be an antibody to an endogenous BBB receptor-mediated transporter, as described herein. The peptide that is active in the CNS can be a neurotherapeutic agent, e.g., a neurotrophin.

In some embodiments, the neurotrophin is selected from the group consisting of brain-derived neurotrophic factor, nerve growth factor (NGF), neurotrophin-4/5, fibroblast growth factor (FGF)-2 and other FGFs, neurotrophin (NT)-3, erythropoietin (EPO), hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF)-α, TGF-β, vascular endothelial growth factor (VEGF), interleukin-1 receptor antagonist (IL-1ra), ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), neurturin, platelet-derived growth factor (PDGF), heregulin, neuregulin, artemin, persephin, interleukins, granulocyte-colony stimulating factor (CSF), granulocyte-macrophage-CSF, netrins, cardiotrophin-1, hedgehogs, leukemia inhibitory factor (LIF), midkine, pleiotrophin, bone morphogenetic proteins (BMPs), netrins, saposins, semaphorins, or stem cell factor (SCF). In some embodiments, the neurotrophin is BDNF such as a truncated BDNF, e.g., a carboxyl-truncated BDNF. The carboxyl-truncated BDNF is lacking the two carboxyl terminal amino acids in some embodiments. The structure capable of crossing the BBB and the neurotherapeutic agent are covalently linked by a peptide linker in some embodiments.

[00128]   In certain embodiments, the invention provides compositions that increase the serum half-life of cationic substances. One limitation for many current therapeutics, especially cationic therapeutic peptides (e.g., BDNF) is their rapid clearance from the circulation. The positive charge on the cationic substance, such as cationic peptides, rapidly interacts with negative charges on cell membranes, which triggers an absorptive-mediated endocytosis into the cell, particularly liver and spleen. This is true not only for neurotherapeutics (where rapid clearance means only limited contact with the BBB and thus limited ability to cross the BBB) but for other agents as well, such as cationic import peptides such as the tat peptide, or cationic proteins (e.g. protamine, polylysine, polyarginine) that bind nucleic acids, or cationic proteins such as avidin that bind biotinylated drugs. Surprisingly, fusion compositions of the invention that include a cationic therapeutic peptide covalently linked to an immunoglobulin show greatly enhanced serum half-life compared to the same peptide when it was not covalently part of a fusion immunoglobulin. This is an important finding, because it shows that the fusion of a highly cationic protein, e.g., BDNF, to an immunoglobulin, e.g. HIRMAb, has two important and unexpected effects: 1) it greatly enhances the serum half-life of the cationic protein, and 2) it does not accelerate the blood clearance of the immunoglobulin to which it is attached, e.g., the HIRMAb. Prior work shows that the noncovalent attachment of a cationic therapeutic peptide, e.g., the cationic BDNF to a monoclonal antibody greatly accelerated the blood clearance of the antibody, owing to the cationic nature of the BDNF, which greatly enhances hepatic uptake. The work in Figure 27A and Example 7 shows that when the cationic therapeutic peptide, e.g., BDNF is re-engineered as an IgG fusion protein, the plasma pharmacokinetics is dominated by the IgG moiety, and that the blood level of the BDNF remains high for a prolonged period; indeed, the serum half-life of the BDNF in the fusion protein is at least about 100 times that of the BDNF alone.

[00129]   Accordingly, in some embodiments, the invention provides composition comprising a cationic therapeutic peptide covalently linked to an immunoglobulin, wherein the cationic therapeutic peptide in the composition has a serum half-life that is an average of at least about 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more than about 100-fold greater than the serum half-life of the cationic therapeutic peptide alone. In some embodiments, the invention provides a composition comprising a cationic therapeutic peptide covalently linked to an immunoglobulin, wherein the cationic therapeutic peptide in the composition has a mean residence time (MRT) in the serum that is an average of at least about 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more than about 100-fold greater than the serum half-life of the cationic therapeutic peptide alone. In some embodiments, the invention provides composition comprising a cationic therapeutic peptide covalently linked to an immunoglobulin, wherein the cationic therapeutic peptide in the composition has a systemic clearance rate that is an average of at least about 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more than about 100-fold slower than the systemic clearance rate of the cationic therapeutic peptide alone. In some embodiments, the invention provides composition comprising a cationic therapeutic peptide covalently linked to an immunoglobulin, wherein the cationic therapeutic peptide in the composition has average blood level after peripheral administration that is an average of at least about 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more than about 100-fold greater than the average blood level after peripheral administration of the cationic therapeutic peptide alone.

[00130]   In some embodiments, the cationic therapeutic peptide comprises a neurotherapeutic agent. Examples of neurotherapeutic agents that are cationic peptides interferons, interleukins, cytokines, or growth factors with an isoelectric point (pI) above 8. In some embodiments, the neurotherapeutic agent is a neurotrophin. Cationic peptide neurotrophins include BDNF, GDNF, NT-3, NT-4/5, NGF, and FGF-2. In some embodiments, the neurotrophin is BDNF or GDNF.

[00131]   In some embodiments, the immunoglobulin is an antibody to an endogenous BBB receptor-mediated transport system. In some embodiments, the endogenous BBB receptor-mediated transport system is selected from the group consisting of the insulin BBB transport system, the BBB transferrin receptor, the BBB leptin receptor, the BBB IGF receptor, or the BBB lipoprotein receptor. In some embodiments, the antibody is an antibody to the endogenous insulin BBB receptor-mediated transport system. Antibodies can be any suitable antibody as described herein.

[00132]   Pharmaceutical compositions The invention also provides pharmaceutical compositions that contain one or more compositions of the invention and a pharmaceutically acceptable excipient. A thorough discussion of pharmaceutically acceptable carriers/excipients can be found in *Remington's Pharmaceutical Sciences*, Gennaro, AR, ed., 20th edition, 2000: Williams and Wilkins PA, USA.. Pharmaceutical compostions of the invention include compositions suitable for administration via any peripheral route, including intravenous, subcutaneous, intrmuscular,

intraperitoneal injection; oral, rectal, transbuccal, pulmonary, transdermal, intranasal, or any other suitable route of peripheral administration.

[00133]   The compostions of the invention are particular suited for injection, e.g., as a pharmaceutical composition for intravenous, subcutaneous, intramuscular, or intraperitonal administration. Aqueous compositions of the present invention comprise an effective amount of a composition of the present invention, which may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, e.g., a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[00134]   Exemplary pharmaceutically acceptable carriers for injectable compositions can include salts, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. For example, compositions of the invention may be provided in liquid form, and formulated in saline based aqueous solution of varying pH (5-8), with or without detergents such polysorbate-80 at 0.01-1%, or carbohydrate additives, such mannitol, sorbitol, or trehalose. Commonly used buffers include histidine, acetate, phosphate, or citrate. Under ordinary conditions of storage and use, these preparations can contain a preservative to prevent the growth of microorganisms. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol; phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate, and gelatin.

[00135]   For human administration, preparations meet sterility, pyrogenicity, general safety, and purity standards as required by FDA and other regulatory agency standards. The active compounds will generally be formulated for parenteral administration, e.g., formulated for injection via the intravenous, intramuscular, subcutaneous, intralesional, or intraperitoneal routes. The preparation of an aqueous composition that contains an active component or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for use in preparing solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

[00136]   Sterile injectable solutions are prepared by incorporating the active compounds in the
required amount in the appropriate solvent with various of the other ingredients enumerated above,
as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating
the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion
medium and the required other ingredients from those enumerated above. In the case of sterile
powders for the preparation of sterile injectable solutions, methods of preparation include vacuum-
drying and freeze-drying techniques which yield a powder of the active ingredient plus any
additional desired ingredient from a previously sterile-filtered solution thereof.

[00137]   Upon formulation, solutions will be administered in a manner compatible with the dosage
formulation and in such amount as is therapeutically effective. The formulations are easily
administered in a variety of dosage forms, such as the type of injectable solutions described above,
but drug release capsules and the like can also be employed

[00138]   The term "unit dose" refers to physically discrete units suitable for use in a subject, each
unit containing a predetermined-quantity of the therapeutic composition calculated to produce the
desired responses, discussed above, in association with its administration, i.e., the appropriate route
and treatment regimen. The quantity to be administered, both according to number of treatments
and unit dose, depends on the subject to be treated, the state of the subject and the protection
desired. The person responsible for administration will, in any event, determine the appropriate
dose for the individual subject.

[00139]   The active therapeutic agents may be formulated within a mixture to comprise about
0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 1.0 to 100 milligrams or even
about 0.01 to 1.0 grams per dose or so. Multiple doses can also be administered. In some
embodiments, a dosage of about 2.5 to about 25 mg of a fusion protein of the invention is used as a
unit dose for administration to a human, e.g., about 2.5 to about 25 mg of a fusion protein of GDNF
and a HIR MAb.

[00140]   In addition to the compounds formulated for parenteral administration, such as intravenous
or intramuscular injection, other alternative methods of administration of the present invention may
also be used, including but not limited to intradermal administration (See U.S. Pat. Nos. 5,997,501;
5,848,991; and 5,527,288), pulmonary administration (See U.S. Pat. Nos. 6,361,760; 6,060,069;
and 6,041,775), buccal administration (See U.S. Pat. Nos. 6,375,975; and 6,284,262), transdermal
administration (See U.S. Pat. Nos. 6,348,210; and 6,322,808) and transmucosal administration (See
U.S. Pat. No. 5,656,284). All such methods of administration are well known in the art. One may
also use intranasal administration of the present invention, such as with nasal solutions or sprays,
aerosols or inhalants. Nasal solutions are usually aqueous solutions designed to be administered to
the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many
respects to nasal secretions. Thus, the aqueous nasal solutions usually are isotonic and slightly
buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those
used in ophthalmic preparations and appropriate drug stabilizers, if required, may be included in the

formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

[00141] Additional formulations, which are suitable for other modes of administration, include suppositories and pessaries. A rectal pessary or suppository may also be used. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum or the urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. For suppositories, traditional binders and carriers generally include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in any suitable range, e.g., in the range of 0.5% to 10%, preferably 1%-2%.

[00142] Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders. In certain defined embodiments, oral pharmaceutical compositions will comprise an inert diluent or assimilable edible carrier, or they may be enclosed in a hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations can contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied, and may conveniently be between about 2 to about 75% of the weight of the unit, or between about 25-60%. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

[00143] The tablets, troches, pills, capsules and the like may also contain the following: a binder, such as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent, methylene and propyl parabens as preservatives, a dye and flavoring, such as cherry or orange flavor. In some embodiments, an oral pharmaceutical composition may be enterically coated to protect the active ingredients from the environment of the stomach; enteric coating methods and formulations are well-known in the art.

VI. Nucleic acids, vectors, cells, and manufacture.

[00144]   The invention also provides nucleic acids, vectors, cells, and methods of production.

A. Nucleic acids

[00145]   In some embodiments, the invention provides nucleic acids that code for proteins or peptides of the invention. In certain embodiments, the invention provides a single nucleic acid sequence containing a first sequence coding for a light chain of an immunoglobulin and second sequence coding a heavy chain of the immunoglobulin, where either the first sequence also codes for a peptide that is expressed as a fusion protein of the peptide covalently linked to the light chain, or the second sequence also codes for a peptide that is expressed as a fusion protein of the peptide covalently linked to the heavy chain. In some embodiments, the invention provides nucleic acid sequences, and in some embodiments the invention provides nucleic acid sequences that are at least about 60, 70, 80, 90, 95, 99, or 100% identical to a particular nucleotide sequence. For example, in some embodiments, the invention provides a nucleic acid containing a first sequence that is at least about 60, 70, 80, 90, 95, 99, or 100% identical to SEQ ID NO:45and a second sequence that is at least about 60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 1396-1746 of SEQ ID NO: 33.

[00146]   In other embodiments, the invention provides a nucleic acid containing a sequence that is at least about 60, 70, 80, 90, 95, 99, or 100% identical to SEQ ID NO:45.

[00147]   For sequence comparison, of two nucleic acids, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[00148]   A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, including but not limited to, by the local homology algorithm of Smith and Waterman (1970) *Adv. Appl. Math.* 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Nat'l. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g.,* Ausubel *et al., Current Protocols in Molecular Biology* (1995 supplement)).

[00149]   One example of an algorithm that is suitable for determining percent sequence identity and
sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et
al.* (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410,
respectively. Software for performing BLAST analyses is publicly available through the National
Center for Biotechnology Information. The BLAST algorithm parameters W, T, and X determine
the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses
as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both
strands. The BLAST algorithm is typically performed with the "low complexity" filter turned off.
The BLAST algorithm also performs a statistical analysis of the similarity between two sequences
(*see, e.g.*, Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of
similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides
an indication of the probability by which a match between two nucleotide or amino acid sequences
would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if
the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is
less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[00150]   The invention provides nucleic acids that code for any of the peptides of the invention. In
some embodiments, the invention provides a single nucleic acid sequence containing a gene coding
for a light chain of an immunoglobulin and a gene coding for a fusion protein, where the fusion
protein includes a heavy chain of the immunoglobulin covalently linked to a peptide. In some
embodiments, the peptide is a therapeutic peptide. In some embodiments the peptide is a
neurotherapeutic peptide, e.g., a neurotrophin such as BDNF or GDNF (e.g., mature human
GDNF). In some embodiments, the BDNF is a two amino acid carboxy-truncated BDNF. In some
embodiments, the immunoglobulin is an IgG. In some embodiments, the IgG is a MAb, such as a
chimeric MAb. The antibody can be an antibody to a transport system, e.g., an endogenous BBB
receptor-mediated transport system such as the endogenous BBB receptor-mediated insulin
transport system. In some embodiments, the endogenous BBB receptor-mediated insulin transport
system is a human endogenous BBB receptor-mediated insulin transport system and wherein the
peptide to which the immunoglobulin heavy chain is covalently linked is human BDNF or human
GDNF. Any suitable peptide, neurotherapeutic peptide, neurotrophin, BDNF, GDNF (e.g., mature
human GDNF), antibody, monoclonal antibody, or chimeric antibody, as described herein, may be
coded for by the nucleic acid, combined as a fusion protein and coded for in a single nucleic acid
sequence. As is well-known in the art, owing to the degeneracy of the genetic code, any
combination of suitable codons may be used to code for the desired fusion protein. In addition,
other elements useful in recombinant technology, such as promoters, termination signals, and the
like, may also be included in the nucleic acid sequence. Such elements are well-known in the art.
In addition, all nucleic acid sequences described and claimed herein include the complement of the
sequence.

[00151]   In some embodiments the nucleic acid codes for a BDNF, e.g., a variant BDNF, or GDNF
(e.g., mature human GDNF) as a component of the fusion protein, which also comprises an
immunoglobulin sequence. In some embodiments, the BDNF contains a sequence that is about 60,
70,80, 90, 95, 99, or 100% identical to the sequence of amino acids 466-582 of SEQ ID NO: 24. In
some embodiments, the GDNF contains a sequence that is at least about 60, 70,80, 90, 95, 99, or
100% identical to the sequence of SEQ ID NO:52. In some embodiments, the amino acid sequence
of the encoded GDNF consists essentially of SEQ ID NO:52. In some embodiments, the nucleic
acid codes for a fusion protein comprising an amino acid sequence that is at least about 60, 70,80,
90, 95, 99, or 100% identical to the sequence of SEQ ID NO:46. In some embodiments, the
encoded nucleic acid comprises the amino acid sequence of SEQ ID NO:46. In some embodiments,
the BDNF or GDNF is linked at its amino terminus to carboxy terminus of the heavy chain of the
immunoglobulin, e.g., MAb. The heavy chain of the MAb can comprise a sequence that is about
60, 70, 80, 90, 95, 99 or 100% identical to amino acids 20-462 of SEQ ID NO: 24. In some
embodiments, the light chain of the immunoglobulin, e.g., MAb, comprises a sequence that is about
60, 70, 80, 90, 95, 99 or 100% identical to amino acids 21-234 of SEQ ID NO: 36. The nucleic
acid can further contain a nucleic acid sequence that codes for a peptide linker between the heavy
chain of the MAb and the BDNF or GDNF. In some embodiments, the linker is S-S-M. The
nucleic acid may further contain a nucleic acid sequence coding for a signal peptide, wherein the
signal peptide is linked to the heavy chain. Any suitable signal peptide, as known in the art or
subsequently developed, may be used. In some embodiments, the signal peptide attached to the
heavy chain comprises a sequence that is about 60, 70, 80, 90, 95, 99, or 100% identical to amino
acids 1-19 of SEQ ID NO: 24. In some embodiments, the nucleic acid contains a nucleic acid
sequence coding for another signal peptide, wherein the other signal peptide is linked to the light
chain. The signal peptide linked to the light chain can comprise a sequence that is about 60, 70, 80,
90, 95, 99, or 100% identical to amino acids 1-20 of SEQ ID NO: 36. The nucleic acid can contain
a nucleic acid sequence coding for a selectable marker. In some embodiments the selectable marker
is DHFR. The sequence of the DHFR can be about 60, 70, 80, 90, 95, 99, or 100% identical to
amino acids 1-187 of SEQ ID NO: 38.

[00152]   In certain embodiments, the invention provides a nucleic acid comprising a first sequence
that codes for a neurotherapeutic peptide, e.g., a neurotrophin such as BDNF, in the same open
reading frame as a second sequence that codes for an immunoglobulin component. The
immunoglobulin component can be, e.g., a light chain or a heavy chain, e.g., that is at least about
60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 58-1386-of SEQ ID NO: 33 and a second
sequence that is at least about 60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 1396-1746 of
SEQ ID NO: 33. In some embodiments, the nucleic acid also contains a third sequence that is at
least about 60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 61-702 of SEQ ID NO: 35. In
some embodiments, the nucleic acid further contains a fourth sequence that codes for a first signal
peptide and a fifth sequence that codes for a second signal peptide. In some embodiments, the

fourth sequence is at least about 60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 1-57 of SEQ ID NO: 33 and the fifth sequence is at least about 60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 1-60 of SEQ ID NO: 35. In some embodiments, the nucleic acid further contains a sequence that codes for a selectable marker, such as dihydrofolate reductase (DHFR). In some embodiments, the sequence that codes for the DHFR is at least about 60, 70, 80, 90, 95, 99, or 100% identical to nucleotides 1-561 of SEQ ID NO: 37.

B. Vectors

[00153]   The invention also provides vectors. The vector can contain any of the nucleic acid sequences described herein. In some embodiments, the invention provides a single tandem expression vector containing nucleic acid coding for an antibody heavy chain fused to a peptide, e.g., a therapeutic peptide such as a neurotrophin, and nucleic acid coding for a light chain of the antibody, all incorporated into a single piece of nucleic acid, e.g., a single piece of DNA. The single tandem vector can also include one or more selection and/or amplification genes. A method of making an exemplary vector of the invention is provided in the Examples. However, any suitable techniques, as known in the art, may be used to construct the vector.

[00154]   The use of a single tandem vector has several advantages over previous techniques. The transfection of a eukaryotic cell line with immunoglobulin G (IgG) genes generally involves the co-transfection of the cell line with separate plasmids encoding the heavy chain (HC) and the light chain (LC) comprising the IgG. In the case of a IgG fusion protein, the gene encoding the recombinant therapeutic protein may be fused to either the HC or LC gene. However, this co-transfection approach makes it difficult to select a cell line that has equally high integration of both the HC and LC-fusion genes, or the HC-fusion and LC genes. The approach to manufacturing the fusion protein utilized in certain embodiments of the invention is the production of a cell line that is stably transfected with a single plasmid DNA that contains all the required genes on a single strand of DNA, including the HC-fusion protein gene, the LC gene, the selection gene, e.g. neo, and the amplification gene, e.g. the dihydrofolate reductase gene. As shown in the diagram of the fusion protein tandem vector in Figure 12, the HC-fusion gene, the LC gene, the neo gene, and the DHFR gene are all under the control of separate, but tandem promoters and separate but tandem transcription termination sequences. Therefore, all genes are equally integrated into the host cell genome, including the fusion gene of the therapeutic protein and either the HC or LC IgG gene.

C. Cells

[00155]   The invention further provides cells that incorporate one or more of the vectors of the invention. The cell may be a prokaryotic cell or a eukaryotic cell. In some embodiments, the cell is a eukaryotic cell. In some embodiments, the cell is a mouse myeloma hybridoma cell. In some embodiments, the cell is a Chinese hamster ovary (CHO) cell. Exemplary methods for incorporation of the vector(s) into the cell are given in the Examples. However, any suitable techniques, as known in the art, may be used to incorporate the vector(s) into the cell. In some embodiments, the invention provides a cell capable of expressing an immunoglobulin fusion

protein, where the cell is a cell into which has been introduced a single tandem expression vector, where both the immunoglobulin light chain gene and the gene for the immunoglobulin heavy chain fused to the therapeutic agent, are incorporated into a single piece of nucleic acid, e.g., DNA. In some embodiments, the invention provides a cell capable of expressing an immunoglobulin fusion protein, where the cell is a cell into which has been stably transfected a single tandem expression vector, where both the immunoglobulin heavy chain gene and the gene for the immunoglobulin light chain fused to the therapeutic agent, are incorporated into a single piece of nucleic acid, e.g., DNA. The introduction of the tandem vector may be by, e.g., permanent integration into the chromsomal nucleic acid, or by, e.g., introduction of an episomal genetic element.

D. Methods of manufacture

[00156]   In addition, the invention provides methods of manufacture. In some embodiments, the invention provides a method of manufacturing an immunoglobulin fusion protein, where the fusion protein contains an immunoglobulin heavy chain fused to a therapeutic agent, by introducing into a eukaryotic cell a single tandem expression vector, where both the immunoglobulin light chain gene and the gene for the immunoglobulin heavy chain fused to the therapeutic agent, are incorporated into a single piece of nucleic acid, e.g., DNA. In some embodiments, the invention provides a method of manufacturing an immunoglobulin fusion protein, where the fusion protein contains an immunoglobulin light chain fused to a therapeutic agent, by ly introducing into a eukaryotic cell a single tandem expression vector, where both the immunoglobulin heavy chain gene and the gene for the immunoglobulin light chain fused to the therapeutic agent, are incorporated into a single piece of nucleic acid, e.g., DNA. In some embodiments, the introduction of the vector is accomplished by integration into the host cell genome. In some embodiments, the introduction of the vector is accomplished by introduction of an episomal genetic element containing the vector into the host cell. Episomal genetic elements are well-known in the art In some embodiments, the therapeutic agent is a neurotherapeutic agent. In some embodiments, the single piece of nucleic acid further includes one or more genes for selectable markers. In some embodiments, the single piece of nucleic acid further includes one or more amplification genes. In some embodiments, the immunoglobulin is an IgG, e.g., a MAb such as a chimeric MAb. The methods may further include expressing the immunoglobulin fusion protein, and/or purifying the immunoglobulin fusion protein. Exemplary methods for manufacture, including expression and purification, are given in the Examples.

[00157]   However, any suitable techniques, as known in the art, may be used to manufacture, optionally express, and purify the proteins. These include non-recombinant techniques of protein synthesis, such as solid phase synthesis, manual or automated, as first developed by Merrifield and described by Stewart et al. in *Solid Phase Peptide Synthesis* (1984). Chemical synthesis joins the amino acids in the predetermined sequence starting at the C-terminus. Basic solid phase methods require coupling the C-terminal protected α-amino acid to a suitable insoluble resin support. Amino

acids for synthesis require protection on the α-amino group to ensure proper peptide bond formation with the preceding residue (or resin support). Following completion of the condensation reaction at the carboxyl end, the α-amino protecting group is removed to allow the addition of the next residue. Several classes of α-protecting groups have been described, see Stewart et al. in *Solid Phase Peptide Synthesis* (1984), with the acid labile, urethane-based tertiary-butyloxycarbonyl (Boc) being the historically preferred. Other protecting groups, and the related chemical strategies, may be used, including the base labile 9-fluorenylmethyloxycarbonyl (FMOC). Also, the reactive amino acid sidechain functional groups require blocking until the synthesis is completed. The complex array of functional blocking groups, along with strategies and limitations to their use, have been reviewed by Bodansky in *Peptide Synthesis* (1976) and, Stewart et al. in *Solid Phase Peptide Synthesis* (1984).

[00158]   Solid phase synthesis is initiated by the coupling of the described C-terminal α-protected amino acid residue. Coupling requires activating agents, such as dicyclohexycarbodiimide (DCC) with or without 1-hydroxybenzo-triazole (HOBT), diisopropylcarbodiimide (DIIPC), or ethyldimethylaminopropylcarbodiimide (EDC). After coupling the C-terminal residue, the α-amino protected group is removed by trifluoroacetic acid (25% or greater) in dichloromethane in the case of acid labile tertiary-butyloxycarbonyl (Boc) groups. A neutralizing step with triethylamine (10%) in dichloro-methane recovers the free amine (versus the salt). After the C-terminal residue is added to the resin, the cycle of deprotection, neutralization and coupling, with intermediate wash steps, is repeated in order to extend the protected peptide chain. Each protected amino acid is introduced in excess (three to five fold) with equimolar amounts of coupling reagent in suitable solvent. Finally, after the completely blocked peptide is assembled on the resin support, reagents are applied to cleave the peptide form the resin and to remove the side chain blocking groups. Anhydrous hydrogen fluoride (HF) cleaves the acid labile tertiary-butyloxycarbonyl (Boc) chemistry groups. Several nucleophilic scavengers, such as dimethylsulfide and anisole, are included to avoid side reactions especially on side chain functional groups.

VII. Methods

[00159]   The invention also provides methods. In some embodiments, the invention provides methods for transport of an agent active in the CNS across the BBB in an effective amount. In some embodiments, the invention provides therapeutic, diagnostic, or research methods. Diagnostic methods include the development of peptide radiopharmaceuticals capable of transport across the BBB, such as the fusion of a peptide ligand, or peptidomimetic MAb for an endogenous receptor in the brain, followed by the radiolabelling of the fusion protein, followed by systemic administration, and external imaging of the localization within the brain of the peptide radiopharmaceutical.

[00160]   Prior to the present invention, neurotrophins such as BDNF or GDNF were injected directly into the brain to achieve a therapeutic effect, because the neurotrophin does not cross the BBB. Therefore, it is not expected that neurotrophic factors will have beneficial effects on brain disorders following the peripheral (intravenous, subcutaneous) administration of these molecules.

[00161]   However, neurotherapeutics can be developed as drugs for peripheral routes of administration, providing the neurotherapeutic is enabled to cross the BBB. Attachment of the neurotherapeutic, e.g. a neurotrophin such as BDNF or GDNF to a MTH, e.g., the chimeric HIRMAb, offers a new approach to the non-invasive delivery of neurotherapeutics to the CNS in animals, e.g., mammals such as humans for the treatment of acute brain and spinal cord conditions, such as focal brain ischemia, global brain ischemia, and spinal cord injury, and chronic treatment of neurodegenerative disease, including prion diseases, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), ALS, multiple sclerosis, transverse myelitis, motor neuron disease, Pick's disease, addiction (e.g., drug addiction), tuberous sclerosis, lysosomal storage disorders, Canavan's disease, Rett's syndrome, spinocerebellar ataxias, Friedreich's ataxia, optic atrophy, and retinal degeneration.

[00162]   Accordingly, in some embodiments the invention provides methods of transport of an agent active in the CNS from the peripheral circulation across the BBB in an effective amount, where the agent is covalently attached to a structure that crosses the BBB, and where the agent alone is not transported across the BBB in an effective amount. In some embodiments the invention provides methods of transport of neurotherapeutic agent from the peripheral circulation across the BBB in a therapeutically effective amount, where the neurotherapeutic agent is covalently attached to a structure that crosses the BBB, and where the neurotherapeutic agent alone is not transported across the BBB in a therapeutically effective amount.

[00163]   The invention also provides, in some embodiments, methods of treatment of disorders of the CNS by peripheral administration of an effective amount of a therapeutic agent, e.g., a neurotherapeutic agent covalently linked to a structure that is capable of crossing the BBB, where the agent alone is not capable of crossing the BBB in an effective amount when administered peripherally. In some embodiments, the CNS disorder is an acute disorder, and, in some cases, may require only a single administration of the agent. In some embodiments, the CNS disorder is a chronic disorder and may require more than one administration of the agent.

[00164]   In some embodiments, the effective amount, e.g., therapeutically effective amount is such that a concentration in the brain is reached of at least about 0.001, 0.01, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, or more than 100 ng/gram brain. In some embodiments, a therapeutically effective amount, e.g., of a neurotrophin such as BDNF or GDNF, is such that a brain level is achieved of about 0.1 to 1000, or about 1-100, or about 5-50 ng/g brain. In some embodiments, the neurotherapeutic agent is a neurotrophin. In some embodiments, the neurotrophin is selected from the group consisting of BDNF, nerve growth factor (NGF), neurotrophin-4/5, fibroblast growth factor (FGF)-2 and other FGFs, neurotrophin (NT)-3,

erythropoietin (EPO), hepatocyte growth factor (HGF), epidermal growth factor (EGF),
transforming growth factor (TGF)-α, TGF-β, vascular endothelial growth factor (VEGF),
interleukin-1 receptor antagonist (IL-1ra), ciliary neurotrophic factor (CNTF), glial-derived
neurotrophic factor (GDNF), neurturin, platelet-derived growth factor (PDGF), heregulin,
neuregulin, artemin, persephin, interleukins, granulocyte-colony stimulating factor (CSF),
granulocyte-macrophage-CSF, netrins, cardiotrophin-1, hedgehogs, leukemia inhibitory factor
(LIF), midkine, pleiotrophin, bone morphogenetic proteins (BMPs), netrins, saposins, semaphorins,
or stem cell factor (SCF).. In some embodiments, the neurotrophin is BDNF, e.g. a truncated
BDNF, such as the carboxyl-truncated BDNFs described herein.

[00165]   In some embodiments, the invention provides methods of treating a disorder of the CNS
by peripherally administering to an individual in need of such treatment an effective amount of a
neurotrophin, where the neurotrophin is capable of crossing the BBB to produce an average
elevation of neurotrophin concentration in the brain of at least about 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,
15, 20, 25, 30, 35, 40, 45, 50, 100, or more than 100 ng/gram brain following said peripheral
administration, and where the neurotrophin remains at the elevated level for about 1, 2, 3, 4, 5, 6, 7,
8, 9, 10, or more than 10 days after a single administration. In some embodiments, the neurotrophin
remains at a level of greater than about 1 ng/g brain, or about 2 ng/g brain, or about 5 ng/g brain for
about 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 days after a single administration. In some
embodiments, the neurotrophin is BDNF, including truncated versions thereof.

[00166]   In some embodiments, the invention provides methods of treating a disorder of the CNS
by peripherally administering to an individual in need of such treatment an effective amount of a
composition of the invention. The term "peripheral administration," as used herein, includes any
method of administration that is not direct administration into the CNS, i.e., that does not involve
physical penetration or disruption of the BBB. "Peripheral administration" includes, but is not
limited to, intravenous intramuscular, subcutaneous, intraperitoneal, intranasal, transbuccal,
transdermal, rectal, transalveolar (inhalation), or oral administration. Any suitable composition of
the invention, as described herein, may be used. In some embodiments, the composition is a
neurotrophin covalently linked to a chimeric HIR-MAb. In some embodiments, the neurotrophin is
a BDNF. In some embodiments, the BDNF is a variant as described herein, such as a carboxyl-
terminal truncated variant. In other embodiments, the neurotrophin is a GDNF (e.g., mature human
GDNF).

[00167]   A "disorder of the CNS" or "CNS disorder," as those terms are used herein, encompasses
any condition that affects the brain and/or spinal cord and that leads to suboptimal function. In
some embodiments, the disorder is an acute disorder. Acute disorders of the CNS include focal
brain ischemia, global brain ischemia, brain trauma, spinal cord injury, acute infections, status
epilepticus, migrane headache, acute psychosis, suicidal depression, and acute anxiety/phobia. In
some embodiments, the disorder is a chronic disorder. Chronic disorders of the CNS include
chronic neurodegeneration, retinal degeneration, depression, chronic affective disorders, lysosmal

storage disorders, chronic infections of the brain, brain cancer, stroke rehabilitation, inborn errors of metabolism, autism, mental retardation. Chronic neurodegeneration includes neurodegenerative diseases such as prion diseases, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), transverse myelitis, motor neuron disease, Pick's disease, tuberous sclerosis, lysosomal storage disorders, Canavan's disease, Rett's syndrome, spinocerebellar ataxias, Friedreich's ataxia, optic atrophy, and retinal degeneration, and aging of the CNS.

[00168]   In some embodiments, the invention provides methods of treatment of the retina, or for treatment or prevention of blindness. The retina, like the brain, is protected from the blood by the blood-retinal barrier (BRB). The insulin receptor is expressed on both the BBB and the BRB, and the HIRMAb has been shown to deliver therapeutics to the retina via RMT across the BRB. BDNF is neuroprotective in retinal degeneration, but it was necessary to inject the neurotrophin directly into the eyeball, because BDNF does not cross the BRB. In some embodiments, fusion proteins of the invention are used to treat retinal degeneration and blindness with a route of administration no more invasive than an intravenous or subcutaneous injection, because the HIRMAb delivers the BDNF across the BRB, so that the neurotrophin is exposed to retinal neural cells from the blood compartment.

[00169]   In some embodiments, the invention provides a method of treatment for depression. A subset of patients with depression may have a brain deficiency of BDNF, and the correlation of single nucleotide polymorphisms (SNPs) with affective disorders has been reported. The direct injection of BDNF into the brain has durable anti-depressant effects in rodent model. The BDNF must be injected directly into the brain, because the neurotrophin does not cross the BBB. In some embodiments, the invention provides a method for treating depression by chronic administration of a fusion protein of the invention, thus elevating the brain levels of BDNF and being therapeutic in those patients with depression and a reduced production of brain BDNF.

[00170]   Formulations and administration. Any suitable formulation, route of administration, and dose of the compositions of the invention may be used. Formulations, doses, and routes of administration are determined by those of ordinary skill in the art with no more than routine experimentation. Compositions of the invention, e.g., GDNF fusion proteins are typically administered in a single dose, e.g., an intravenous dose, of about 0.01-1000 mg, or about 0.05-500 mg, or about 0.1-100 mg, or about 1-100 mg, or about 0.5-50 mg, or about 5-50 mg, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 25, 40, 45, 50, 60, 70, 80, 90, or 100 mg. Typically, for the treatment of acute brain disease, such as stroke, cardiac arrest, spinal cord injury, or brain trauma, higher doses may be used, whereas for the treatment of chronic conditions such as Alzheimer's disease, Parkinson's disease, Huntington's disease, MS, ALS, transverse myelitis, motor neuron disease, Pick's disease, tuberous sclerosis, addiction (e.g., drug addiction), lysosomal storage disorders, Canavan's disease, Rett's syndrome, spinocerebellar ataxias, Friedreich's ataxia, optic atrophy, and retinal degeneration, and aging, lower, chronic dosing may be used. Oral

administration can require a higher dosage than intravenous or subcutaneous dosing, depending on the efficiency of absorption and possible metabolism of the protein, as is known in the art, and may be adjusted from the foregoing based on routine experimentation.

[00171]   For intravenous or subcutaneous administration, formulations of the invention may be provided in liquid form, and formulated in saline based aqueous solution of varying pH (5-8), with or without detergents such polysorbate-80 at 0.01-1%, or carbohydrate additives, such mannitol, sorbitol, or trehalose. Commonly used buffers include histidine, acetate, phosphate, or citrate.

[00172]   Dosages for humans can be calculated from appropriate animal data. For example, human dosing of a BDNF-MAB conjugate is based on pre-clinical pharmacokinetic studies, and these measurements have been performed in 2 species, rats, and Rhesus monkeys. Prior work in 3 models of cerebral ischemia in rats demonstrated the range of effective doses of the BDNF-MAb conjugate is 5-50 µg/rat or 20-200 µg/kg of BDNF in the form of the BDNF-MAb conjugate. Since the BDNF component of the fusion protein molecule is 16%, and the HIRMAb component is 84%, the dose of fusion protein is 6-fold greater than the equivalent BDNF dose. Pharmacokinetic studies in rats show these doses produce a concentration of the BDNF in the form of conjugate in plasma of 50-500 ng/mL, and in brain of 5-50 ng/g. Pharmacokinetic studies in adult Rhesus monkeys with the HIRMAb show that the average plasma concentration in the first hour is 0.1% injected dose (ID)/mL, and that the brain concentration is 0.02% ID/g. The brain concentration of the fusion protein is about 0.01% ID/g (Figure 27). Owing to the scaling effect between species, and to the 10-fold larger body size and brain size of humans relative to Rhesus monkeys, the projected plasma and brain concentrations in humans are 0.01% ID/mL and 0.001% ID/g respectively. Since the human brain is 1200 grams, then >1% of the injected dose is delivered to the human brain, which is a level of brain uptake comparable to small molecules. Given an injected dose of fusion protein of 2.5-25 mg in humans, the expected 60 min plasma concentration is 250-2500 ng/ml of fusion protein, and the expected 60 min brain concentration is 25-250 ng/g of fusion protein, which is equivalent to 4-40 ng/gram brain of BDNF. The 5 mg and 25 mg fusion protein doses in humans will produce a brain concentration of the BDNF that is neuroprotective in either global or regional brain ischemia. Since the BDNF comprises 16% of the fusion protein, the effective doses of BDNF administered to humans is 0.4 or 4.0 mg, respectively, for the 2.5 or 25 mg dose of fusion protein.

[00173]   The fusion protein may also be formulated for chronic use for the treatment of a chronic CNS disorder, e.g., neurodegenerative disease, stroke or brain/spinal cord injury rehabilitation, or depression. Chronic treatment may involve daily, weekly, bi-weekly administration of the composition of the invention, e.g., fusion protein either intravenously, intra-muscularly, or subcutaneous in formulations similar to that used for acute treatment. Alternatively, the composition, e.g., fusion protein may be formulated as part of a bio-degradable polymer, and administered on a monthly schedule.

[00174]   Combination therapies. The composition of the invention, e.g., fusion protein may be
administered as part of a combination therapy. The combination therapy involves the
administration of a composition of the invention in combination with another therapy for the CNS
disorder being treated. If the composition of the invention is used in combination with another
CNS disorder method or composition, any combination of the composition of the invention and the
additional method or composition may be used. Thus, for example, if use of a composition of the
invention is in combination with another CNS disorder treatment agent, the two may be
administered simultaneously, consecutively, in overlapping durations, in similar, the same, or
different frequencies, etc. In some cases a composition will be used that contains a composition of
the invention in combination with one or more other CNS disorder treatment agents.

[00175]   Other CNS disorder treatment agents that may be used in methods of the invention
include, without limitation, thromolytic therapy for stroke, amyloid-directed therapy for Alzheimers
disease, dopamine restoration therapy for Parkinsons disease, RNA interference therapy for genetic
disorders, cancer, or infections, and anti-convulsant therapy for epilepsy. Dosages, routes of
administration, administration regimes, and the like for these agents are well-known in the art.

[00176]   In some embodiments, the composition, e.g., fusion protein is co-administered to the
patient with another medication, either within the same formulation or as a separate composition.
For example, the fusion protein could be formulated with another fusion protein that is also
designed to deliver across the human blood-brain barrier a recombinant protein other than BDNF
(e.g., GDNF). The fusion protein may be formulated in combination with other large or small
molecules.

VIII. Kits

[00177]   Compositions of the invention, e.g., fusion proteins, may be provided as a kit that includes
the formulation, e.g., fusion protein in a container and in suitable packaging. The composition can
be provided in a dry powder form, in solid form (i.e., lyophilized), in solution, or in suspension. If
the composition is a protein, to the proteins may have been added emulsifiers, salts, preservatives,
other proteins, nucleic acids, protease inhibitors, antibiotics, perfumes, polysaccharides, adhesive
agents, polymers, microfibrils, oils, etc. The composition is packaged for transport, storage and/or
use by a consumer. Such packaging of therapeutic compositions for transport, storage, and use is
well-known in the art. Packaged compositions may include further components for the dispensing
and storage of the composition, and may also include separately packaged diluent comprised of,
e.g., sterile water or a suitable buffer, for solubilizing the formulation, e.g., fusion protein prior to
administration to the patient. Kits of the invention may also include written materials, including
instructions for use, results of clinical studies, desired outcome and expected course of treatment,
information about precautions and side effects, and the like. The kits may optionally further
contain other components, such as gloves, scissors, tape, implements for disposal of used vials and
other waste, masks, antiseptic, antibiotics, and the like.

EXAMPLES

Example 1

Construction of the single tandem vector containing complete genes for IgG-neurotherapeutic fusion

[00178]   Genetic engineering of a eukaryotic expression vector encoding the heavy chain (HC) of the fusion protein is outlined in Figure 1. The final fusion protein HC expression vector was designated pHIRMAb-BDNF, or clone 416. This vector was designed to produce a fusion protein, comprised of a BDNF variant fused to the HC of the HIRMAb. Either BDNF or a variant of BDNF (vBDNF) can be fused to the HIRMAb. The vBDNF differs from native human BDNF by substitution of certain amino acids, such as a vBDNF where the 2 amino acids at the carboxyl terminus of BDNF are absent in vBDNF. The clone 416 plasmid was derived from clone 400, which produces the HC of the chimeric form of the HIRMAb, and a cDNA encoding mature human vBDNF, which was produced as described in Figure 2. Clone 400 encodes a chimeric human IgG1 that is derived from a chromosomal fragment encoding the human IgG1 constant region, and is comprised of both intron and exon sequences. The HC gene of the chimeric HIRMAb in clone 400 was subcloned at the BamHI site of the pCR II plasmid to facilitate engineering of the stop codon located at the 3'-end of the CH3 region by site directed mutagenesis (SDM). The engineering of the stop codon located at the end of the CH3 region was performed by site-directed mutagenesis to produce a SspI site. The SspI site allows for insertion of the vBDNF cDNA (Figure 3) by blunt-end ligation into clone 400 to form clone 415. SDM was performed using the QuickChange SDM kit (Stratagene, CA). Sense and complementary mutagenic primers were designed in a way that the CH3 stop codon (aaTGAg) is mutated to SspI site (aaTATt). In addition, primers contained 15 nucleotides of the stop codon 5'- and 3'-surrounding region; the sequence of these primers, designated SDM-SspI forward (FWD) and reverse (REV) are given in Table 1.

Table 1

Nucleotide sequence of oligodeoxynucleotides used for engineering plasmid clone 416

SDM-SspI-FWD (SEQ ID NO. 1)

CCTGTCTCCGGGTAAATATTTGCGACGGCCGGCAAG

SDM-SspI-REV (SEQ ID NO. 2)

CTTGCCGGCCGTCGCAAATATTTACCCGGAGACAGG

XhoI-NheI linker FWD (SEQ ID NO. 3)

ATGCTCGAGGAATTCCCATGGATGATGGCTAGCAAGCTTATG

XhoI-NheI linker REV (SEQ ID NO. 4)

CATAAGCTTGCTAGCCATCATCCATGGGAATTCCTCGAGCAT

XhoI-NheI (underlined) is a Universal linker that contains the following RE sites: XhoI-EcoRI-NcoI-NheI-HindIII. SDM=site-directed mutagenesis; FWD=forward; REV=reverse

[00179]   DNA sequence analysis of the IgG promoter region revealed the presence of additional SspI sites in this region. Therefore, it was first necessary to release the HC promoter region (PRO-VH) by digestion of clone 404 with XhoI and NheI, and the clone 404 was re-closed with a XhoI-NheI linker which produced clone 405 (-Pro-VH). The sequence of the forward and reverse ODNs used to produce the XhoI-NheI linker are given in Table 1. Plasmid clone 405 (-Pro-VH) now carries the single SspI site introduced by SDM. The human vBDNF cDNA was subcloned at SspI to form an intermediate plasmid named clone 414 (not shown). The complete fusion protein HC expression cassette was then reconstructed by subcloning of the PRO-VH fragment previously deleted to form clone 415. The fusion protein HC gene was then subcloned in the eukaryotic expression vector, clone 400, at the BamHI site to form clone 416.

[00180]   The vBDNF cDNA was produced by PCR via either of 2 equivalent approaches. In one approach, a prokaryotic expression plasmid, pHTBS01, isolated as an expressed sequence tag (EST), and encoding human BDNF, was digested with BamHI and BpII, and gel purified, and re-ligated with T4 ligase and the 5'-end linker to produce clone 412 (Figure 2). The sequence of the forward and reverse ODNs used to produce the 5'-end linker are given in Table 2.

[00181]

Table 2

Engineering of 5'- and 3'-end linkers of vBDNF cDNA

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1) 5'-end linker of vBDNF FWD-ODN (SEQ ID NO. 5)
TCCGGATCCTCGCGAGTATGCACTCTGACCCTGCCCGTCGAGGTGAGCTGAGC GTG

2) 5'-end linker of vBDNF REV-ODN (SEQ ID NO. 6)
CACGCTCAGCTCACCTCGACGGGCAGGGTCAGAGTGCATACTCGCGAGGATCC GGA

3) 3'-end linker of vBDNF FWD-ODN (SEQ ID NO. 7)
AGTCGTACGTGCGGGCCCTTACCATGGATAGCAAAAAGAGAATTGGCTGGCG ATTCATAAGGATAGACACTTCTTGTGTATGTACATTGACCATTAAAAGGTGAT CGCGACTCGAGATG

4) 3'-end linker of vBDNF REV-ODN (SEQ ID NO. 8)

CATCTCGAGTCGCGATCACCTTTTAATGGTCAATGTACATACACAAGAAGTGT
CTATCCTTATGAATCGCCAGCCAATTCTCTTTTTGCTATCCATGGTAAGGGCCC
GCACGTACGACT

5) vBDNF-PCR-U87 FWD-ODN (SEQ ID NO. 9)

ATC<u>TCGCGA</u>GTATGCACTCTGACCCTGCC

6) vBDNF-PCR-U87 REV-ODN (SEQ ID NO. 10)

ATC<u>TCGCGA</u>TCACCTTTTAATGGTCAA

SEQ ID NO 5 and 6: Artificial forward (FWD) and reverse (REV) oligodeoxynucleotide
(ODN) duplex linkers were designed to engineer a mature vBDNF cDNA that allows for
insertion into the CH3 open reading frame (orf) of clone 400 heavy chain (HC) to form
clone 416 (Figure 1). The 5'-end linker is flanked by BamHI and EspI, respectively, and it
reconstructs the amino terminus of the mature vBDNF. BamHI and EspI allow for
directional subcloning into the vBDNF intermediate plasmid clone 413 (Figure 2). A NruI
site follows BamHI and it enables insertion of the vBDNF into the HC vector (clone 405,
Figure 1) at the SspI site. In addition, the linker also has "GT" immediately after NruI to
maintain the orf of the CH3 (Figure 1). This modification introduces a Ser-Ser-Met linker
between CH3 and the vBDNF amino terminus.

SEQ ID NO 7 and 8: The 3'-end linker contains SplI and XhoI to reconstruct the COOH
terminus of the mature vBDNF and introduces a stop codon "TGA". This linker has SplI,
XhoI and NruI sites for directional subcloning and insertion into clone 405 (Figures 1 and
2).

SEQ ID NO 9 and 10: FWD ODN reconstructs the amino terminus of the mature vBDNF
and introduces a Ser-Ser-Met linker. NruI site for insertion into the expression vector is
underlined. REV ODN introduces the TGA stop codon. NruI site for insertion into the
expression vector is underlined.

[00182] Clone 412 was then digested with XhoI and BsiWI, and gel purified, and re-ligated with
T4 ligase and the 3'end linker to produce clone 413 (Figure 2). The sequence of the forward and
reverse ODNs used to produce the 3'-end linker are given in Table 2. The vBDNF cDNA, encoding
the vBDNF with a reconstructed stop codon, was released from clone 413 by NruI, and gel purified;
the ethidium bromide stain of the agarose gel is shown in Figure 3A. This gel shows the expected
size of the vBDNF cDNA, 0.4 kb, and the vector backbone, 3.5 kb. Alternatively, the BDNF
cDNA was produced by PCR from cDNA derived by reverse transcription of polyA+RNA isolated

from human U87 glioma cells, which produce neurotrophins. The primers used to produce the
vBDNF by PCR from the U87-derived cDNA are given in Table 2. This PCR produced the
expected 0.4 kb vBDNF cDNA (Figure 3B). The 0.4 kb vBDNF fragment was then digested with
NruI, and subcloned into clone 415, as described in Figure 1, to produce the full fusion protein HC
expression cassette, which was released by BamHI and subcloned into the original eukaryotic
expression plasmid to produce clone 416 (Figure 1), the final expression plasmid for the fusion
protein HC. Clone 416 was analyzed by double digestion with NheI and BamHI and compared
with that of the original clone 400, which lacks the vBDNF. The agarose gel-separated products are
shown in Figure 3C, where lanes 1 and 3 show the fragments generated from clone 416 and clone
400, respectively. Both plasmids produce a 6 kb vector backbone (upper of 3 bands in lanes 1 and
3), and a 2.5 kb promoter region (lower of 3 bands in lanes 1 and 3). However, the size of the
middle band is 0.4 kb larger for clone 416, as compared to clone 400 (middle band, lanes 1 and 3).
A negative clone is shown in lane 2 of Figure 3C.

[00183]   The nucleotide and amino acid sequence of the reconstructed carboxyl terminus at the
CH3 region of the HIRMAb HC, a 3-amino acid linker (Ser-Ser-Met), the vBDNF sequence,
followed by a stop codon is shown in Figure 4. The entire 2711 nucleotides (nt) comprising the
fusion protein HC gene of clone 416 is shown in Figure 5. The ATG initiation codon and the TGA
stop codon are underlined. The human IgG1 constant region intron and exon sequences are shown
in italics and bold font, respectively, in Figure 5. The vBDNF nt sequence in the clone 416 vector
is underlined in Figure 5. These data show that intronic sequence is found between CH1 and the
hinge region, between the hinge region and CH2, and between CH2 and CH3 regions of the human
IgG1 constant region. The open reading frame (orf) of the fusion protein HC gene encodes for a
563 amino acid protein, following cleavage of a 19 amino acid signal peptide, and the amino acid
sequence of the fusion protein HC is shown in Figure 6. The signal peptide is underlined; the
cysteine (C) residues within the constant region that form inter- or intra-chain disulfide bridges are
shown in bold font; the serine-serine-methionine (SSM) linker between the CH3 region of the IgG
and the vBDNF is underlined; the single N-linked glycosylation site, at the asparagine residue
within CH2 in shown by bold underlined font (Figure 6). The amino acid sequences of the
individual domains of the fusion protein HC protein are given in Figure 7. The vBDNF domain of
the fusion protein is comprised of 117 amino acids.

[00184]   Clone 416 plasmid DNA was electroporated into mouse myeloma cells that had previously
been transfected with an expression plasmid encoding the light chain (LC) of the chimeric
HIRMAb. Since the vBDNF is fused only to the HC, there is no modification of the LC of the
chimeric HIRMAb. Following selection of transfected cell lines, media from 96-well plates were
screened with an ELISA comprised of 2 anti-human IgG antibodies; one antibody is directed
against the heavy chain of human IgG1, and the other antibody is directed against human kappa
light chains. Myeloma clones encoding for intact fusion protein were isolated, and propagated in a
10 L bioreactor. However, the production levels of the fusion protein were low. This low

production was attributed to several factors, including (i) transfection of the myeloma line by 3
separate expression plasmids encoding the heavy chain gene, the light chain gene, and the antibiotic
resistance gene; and (ii) the use of genomic fragment of the heavy and light chain genes with large
intronic sequences. Therefore, the fusion protein expression plasmid was re-engineered with the
following features:

(1)   the polymerase chain reaction (PCR) was used to convert genomic fragments of the fusion
        protein HC and LC genes into 'intron-less' cDNA forms of the 2 genes

(2)   the cDNA forms the fusion protein HC and LC genes were placed on a single 'tandem
        vector' in which the 2 genes were placed in separate and tandem expression cassettes with
        separate promoters

(3)   the promoter driving the expression of the fusion protein HC and LC genes was changed
        from the human IgG promoters to the cytomegalovirus (CMV) promoter, to enable
        transfection of non-myeloma cells, such as Chinese hamster ovary (CHO) cells

(4)   the tandem vector encoding fusion protein contains a gene encoding for the dihydrofolate
        reductase (DHFR) gene, under a separate SV40 promoter, to allow for methotrexate
        (MTX) selection of CHO lines which contain amplification of the genome in the region of
        the insertion of the expression vector.

[00185]   In order to produce the fusion protein tandem vector, it was first necessary to produce
intermediate plasmids, which separately encode cDNA forms of the fusion protein HC and LC
genes. Eukaryotic expression plasmids carrying the CMV promoter and the bovine growth
hormone (BGH) poly-A (pA) transcription termination sequences, and designated pCD, were
digested with NheI and XhoI and re-ligated with T4 ligase and an NheI-EcoRV-KpnI-ScaI-BamHI-
XhoI linker, as shown in Figure 8. The sequence of the forward and reverse ODNs used to produce
this linker are given in Table 3.

Table 3

Nucleotide sequence of ODNs used for engineering of intronless expression vectors

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1) Linker NheI-EcoRV-KpnI-XcaI-BamHI-XhoI FWD ODN (SEQ ID NO. 11)

ATGGCTAGCGATATCGGTACCGTATACGGATCCCTCGAGATG

2) Linker NheI-EcoRV-KpnI-XcaI-BamHI-XhoI REV ODN (SEQ ID NO. 12)

CATCTCGAGGGATCCGTATACGGTACCGATATCGCTAGCCAT

3) PCR cloning of LC FWD ODN primer (SEQ ID NO. 13)

GTGACAAACACAGACATAGGATATC

4) PCR cloning of LC REV ODN primer (SEQ ID NO. 14)

ATGC<u>TCGAG</u>CTAACACTCTCCCCT

<u>5) PCR cloning of fusion protein HC FWD ODN primer</u> (SEQ ID NO. 15)

ATG<u>AATATT</u>CCACCATGGAATGCAGC

<u>6) PCR cloning of fusion protein HC REV ODN primer</u> (SEQ ID NO. 16)

ATA<u>GGATCC</u>TCACCTTTTAATGGTCAA

RE cloning sites are underlined: <u>GATATC</u>: EcoRV, <u>CTCGAG</u>: XhoI, <u>AATATT</u>: SspI,
<u>GGATCC</u>: BamHI.

[00186]   The resulting plasmid, designated pCD-linker (Figure 8) was digested with EcoRV and
BamHI and reclosed with T4 ligase and the fusion protein HC cDNA generated by PCR. For the
PCR reaction, the above mentioned myeloma line that had been dual transfected with genomic
constructs of the fusion protein HC (clone 416) and LC genes were digested and myeloma derived
polyA+ RNA was produced (part A in Figure 8). Oligodeoxythymidine (ODT) primers were used
to produced myeloma cDNA with reverse transcriptase from 0.5 ug of myeloma polyA+RNA,
followed by a final RNase digestion. From this cDNA, PCR was used to produce the cDNA form
of the fusion protein HC gene, using the forward and reverse primers shown in Table 3, and high
fidelity Pfu DNA polymerase. Similarly, the fusion protein LC cDNA was produced by PCR from
the myeloma derived cDNA, and the sequences of the forward and reverse PCR primers used to
amplify the fusion protein LC cDNA are given in Table 3. Following PCR, the cDNA was applied
to an 0.8% agarose gel, and all amplifications yielded a single product, a 1.8 kb fusion protein HC
cDNA (lane 1, Figure 3D), and a 0.7 kb fusion protein LC cDNA (lane 2, Figure 3D). The fusion
protein HC PCR product was digested with SspI and BamHI and subcloned into CD-linker to
produce the clone 422a (Figure 8), which is an intronless eukaryotic expression plasmid encoding
the fusion protein HC cDNA. Clone 422a was analyzed by restriction endonuclease using NheI;
digestion with this enzyme, which has a site in the new multiple cloning region of the pCD vector,
produced the expected 0.4 kb fragment corresponding to the fusion protein heavy chain variable
region (VH) cDNA (lanes 1-4, Figure 3E). The nucleotide sequence of the fusion protein HC
cDNA encoded by clone 422a is shown in Figure 9A, which shows the intron sequences present in
clone 416 (Figure 5) have been deleted by the PCR of processed myeloma RNA. The amino acid
sequence encoded by the fusion protein HC cDNA is given in Figure 9B, and this amino acid
sequence is identical to that produced by the genomic fragment in clone 416 (Figure 6).

[00187]   The fusion protein LC PCR product was digested with EcoRV and XhoI and subcloned
into CD-linker to produce the clone 423a (Figure 10), which is an intronless eukaryotic expression
plasmid encoding the fusion protein LC cDNA. Clone 423a was analyzed by restriction
endonuclease using EcoRV and BamHI; digestion with these enzymes, which have a site in the new
multiple cloning region of the pCD vector, produced the expected 0.7 kb fragment corresponding to
the fusion protein LC cDNA (lanes 1-5, Figure 3F). The nucleotide sequence of the fusion protein
LC cDNA encoded by clone 423a is shown in Figure 11A, which shows the intron sequences have

been deleted by the PCR of processed myeloma RNA. The amino acid sequence encoded by the
fusion protein LC cDNA is shown in Figure 11B.

[00188]   Clones 422a and 423a were the precursors to the fusion protein tandem vector, as outlined
in Figure 12. In 2 steps, clone 422a was subjected to SDM to introduce an EcoRI site at the 3'-end
of the fusion protein HC expression cassette; the sequences of the forward and reverse SDM
primers are given in Table 4.

Table 4

Nucleotide sequences of ODNs used for engineering of TV-12

1) EcoRI-SDM FWD ODN (SEQ ID NO. 17)

AAAAGGCCAGGAACCGAATTCAGATCTCGTTGCTGGCGTTT

2) EcoRI-SDM REV ODN (SEQ ID NO. 18)

AAAACGCCAGCAACGAGATCTGAATTCGGTTCCTGGCCTTT

3) EcoRI linker FWD (SEQ ID NO. 19)

ATCGAATTCAAGCTTGCGGCCGCGTATACAGATCTATC

4) EcoRI linker REV (SEQ ID NO. 20)

GATAGATCTGTATACGCGGCCGCAAGCTTGAATTCGAT

EcoRI site in EcoRI-SDM ODN is underlined.

The EcoRI linker introduces EcoRI-HindIII-NotI-XcaI RE sites.

[00189]   In step 2, the mutated clone 422a was digested with EcoRI, blunt-ended, and re-ligated
with the EcoRI-HindIII-NotI-XcaI linker to produce clone 422a-I (Figure 12). The sequence of the
ODNs used to produce this EcoRI linker are given in Table 4. Clone 422a-I was digested with
EcoRI and HindIII, and closed with T4 ligase in the presence of the fusion protein LC expression
cassette to produce clone 422a-II (Figure 12). The fusion protein LC expression cassette was
generated by digestion of clone pBS-LC-1 with EcoRI and HindIII. Clone pBS-LC-1 was produced
from EcoRV-digested pBS (Bluescript), T4 ligase, and the fusion protein LC expression cassette
produced by digestion of clone 423a with SspI (Figure 12). In parallel, a mouse DHFR expression
cassette, containing the SV40 promoter and the hepatitis C virus polyA region, was produced from
the pFR400 plasmid (designated pDHFR) by digestion of the plasmid with SmaI and SalI
(Figure 12). The final fusion protein tandem vector was produced by subcloning the DHFR
expression cassette into XcaI digested clone 422a-II followed by closure with T4 ligase (Figure 12).
The fusion protein tandem vector was analyzed by restriction endonuclease, and the 11 kb plasmid
was linearized by PvuI (lane 1, Figure 3G). The 1.8 kb fusion protein LC and 1.5 kb DHFR
expression cassettes, and the 8 kb vector backbone including the fusion protein HC expression

cassette were released by digestion with EcoRI and HindIII (lane 2, Figure 3G). The tandem vector
was subjected to DNA sequencing in both directions, and the nucleotide sequence, and the deduced
amino acid sequence of the fusion protein HC, the fusion protein LC, and the DHFR genes are
shown in Figures 14, 15, and 16, respectively. The calculated MW of the fusion protein HC and LC
are 62,220 and 25,760 Da, respectively, not accounting for any carbohydrate content of the fusion
protein HC.

Example 2

[00190]   **Electroporation of CHO cells with fusion protein tandem vector and cultivation in a
bioreactor.** The fusion protein tandem vector (Figure 12) was linearized with PvuI and
electroporated into CHO-K1 cells followed by selection with G418 (375 ug/ml) for 3 weeks.
Positive clones were detected in 96 well plates with a human IgG ELISA that uses 2 primary
antibodies to both the human IgG1 HC and the human kappa LC. Cell lines of high copy number of
the transgene were selected by graded increases in MTX to 600 nM. The MTX-selected cell line
was grown in T175 flasks and then transferred to a 20L bioreactor with a 10L volume of CHO cell
serum free medium (SFM). As shown in Figure 17, the CHO cells were maintained at high density
in excess of 10 million viable cells/mL for nearly 50 days in perfusion mode in the bioreactor. The
secretion by these cells of the fusion protein was detected by ELISA using antibodies to either
human IgG or to human BDNF. As shown in Figure 18, the fusion protein is a 1:1 fusion of the
vBDNF to the carboxyl terminus of the HIRMAb heavy chain, which results in formation of the
fusion protein heavy chain. This heavy chain complexes with the light chain, as shown in
Figure 18. Therefore, the fusion protein should react equally well to 3 antibodies directed against:
(i) the human IgG1 HC, (ii) the human kappa LC; or (iii) human BDNF. As shown in Figure 19,
there is a direct correlation in measurement of the fusion protein in the CHO cell medium
depending on whether anti-human IgG or anti-human BDNF antibodies are used in the ELISA.
These ELISA results were confirmed with immunocytochemistry (ICC), which showed the CHO
cells transfected with TV-120 were immunoreactive with antibodies to either human IgG or to
human BDNF, and that the BDNF immune signal was eliminated by absorption of the anti-BDNF
antibody with recombinant BDNF.

Example 3

[00191]   **Purification and characterization of bioreactor produced fusion protein.** The
conditioned medium obtained from the bioreactor under perfusion mode was passed through a 1 μm
filter, and the medium collected in a 200 L Bioprocess container under sterile conditions, which
were maintained at 4°C in a glass door refrigerator contiguous with the bioreactor. Then, 200 L
batches of conditioned medium were passed through 1 μm and 0.4 μm pre-filters for the removal of
cell debris. The medium was then concentrated with tangential flow filtration (TFF). The TFF
system was a Pellicon 2 model from Millipore and was comprised of five 0.5 m^2 filtration cassettes

with a 30 kDa molecular weight cutoff and a total surface area of 2.5 m^2. A transmembrane gradient of 15 PSI was produced, which results in a reduction in volume of the 200 L to 2 L within 2 hours. The concentrated medium was passed through an 0.22 μ filter prior to elution through 100 mL Prosep A (Millipore) recombinant protein A affinity column. Following application of the sample, the column was washed with buffer A (0.025 M NaCl, 0.025 M Tris, pH=7.4, 3 mM EDTA). The elution of CHO cell host protein (CHOP) was monitored at A280 with a Shimadzu detector. The fusion protein was eluted with 0.1 M citric acid (pH=3) in tubes containing Tris base to cause immediate neutralization to pH 7. The neutralized acid eluate pool was diluted with double distilled water until the conductivity was <7 mS, and the material was applied to a 50 mL Sepharose SP cation exchange column (Amersham) that has been equilibrated with a 0.02 M Tris, pH=7.5. Following washing in the Tris buffer, the residual CHOP was separated from the fusion protein with a linear NaCl gradient from 0 to 1 M NaCl. The fusion protein peak was pooled and buffer exchanged and concentrated with a Millipore diafiltration unit with a 30 kDa molecular weight cutoff. The final concentrated antibody solution was sterile filtered (0.22 μm) and stored at 4°C. The fusion protein was purified to homogeneity on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as demonstrated in Figure 20. The size of the fusion protein heavy chain was 68 kDa as compared to the size of the HIRMAb heavy chain, which was 54 kDa. The difference between the size of the fusion protein and HIRMAb heavy chains reflects the added vBDNF monomer (14 kDa) fused to each heavy chain of the fusion protein. The fusion protein reacts with both anti-human IgG antibodies and anti-human BDNF antibodies on Western blotting with the expected molecular weight size of the immunoreactive bands (Figure 21). Isoelectric focusing (IEF) shows the isoelectric point (pI) of recombinant BDNF was highly cationic with a pI>10 (Figure 22). The observed pI of the fusion protein was 8.5, and approximates the pI of the HIRMAb (Figure 22). The observed pI of the fusion protein, 8.5, was consistent with the calculated pI, which is 9.04 and 5.27 for the fusion protein HC and LC, respectively (http://scansite.mit.edu/).

Example 4

[00192]   The fusion protein is bi-functional and binds with high affinity to both the human insulin receptor and to the human trkB receptor. The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with a competitive ligand binding assay (CLBA) using the lectin affinity purified HIR ECD. CHO cells transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column. The HIR ECD was plated on Nunc-Maxisorb 96 well dishes and the binding of the murine HIRMAb to the HIR ECD was detected by radioactivity measurements following addition of [^{125}I] murine HIRMAb as the ligand in the binding assay (Figure 23A). The binding of the [^{125}I] murine HIRMAb to the HIR ECD was displaced by the addition of unlabeled fusion protein or HIRMAb as demonstrated in Figure 23B. The CLBA shows comparable binding of the HIRMAb or the fusion protein. A Scatchard analysis using a high affinity and low affinity binding site model and

nonlinear regression analysis was performed to determine the affinity constant of the fusion protein binding to the HIR. Both the fusion protein and the HIRMAb bind equally well to the HIR with a high affinity binding constant, Ki=0.63±0.07 nM (Figure 23B).

[00193]   The TrkB CLBA was designed for measurement of the affinity of the fusion protein for recombinant human TrkB ECD. The design of a TrkB CLBA was made difficult by the cationic nature of BDNF, which causes a high degree of nonspecific binding in the assay and this reduces the sensitivity of the assay. The nonspecific binding of BDNF could be eliminated by conjugation of 2000 Da polyethyleneglycol (PEG) to the protein. A bifunctional PEG molecule, biotin-PEG^{2000}-hydrazide (Hz), was commercially obtained, and conjugated to BDNF to produce BDNF-PEG^{2000}-biotin, as outlined in Figure 24A; this molecule was used as the "tracer" in the CLBA. The TrkB ECD was absorbed to ELISA plates and binding of BDNF-PEG^{2000}-biotin to the TrkB was detected colorimetrically with avidin and biotin peroxidase (Figure 24A). Prior studies showed the ELISA signal (A490) was directly proportional to the amount of TrkB added to the well. In addition, the assay had a very low blank and the A490 was <0.04 when no TrkB is plated. The binding of the BDNF-PEG^{2000}-biotin to the TrkB was competitively displaced by the recombinant BDNF (Figure 24B) or the fusion protein (Figure 24C). The Scatchard analysis of the binding data using nonlinear regression analysis allowed for the computation of the Ki of binding of either BDNF or fusion protein to TrkB, as shown in Figure 24B and 24C, respectively. The affinity of the fusion protein for TrkB was not statistically different from the affinity of the recombinant BDNF (Figure 19 B,C). The nonspecific binding (NSB) of the assay was comparable for either BDNF or the fusion protein. The NSB likely represents nonlinear cooperative binding of the neurotrophin to the TrkB extracellular domain. The TrkB CLBA results shown in Figure 24 indicate the affinity of fusion protein for the TrkB receptor was not changed following fusion of the vBDNF to the carboxyl terminus of the HIRMAb heavy chain.

[00194]   Neurotrophins such as BDNF require an obligatory formation of a homo-dimeric structure to be biologically active, and to bind with high affinity to the cognate receptor, e.g. TrkB. A naturally occurring homo-dimeric structure between two BDNF molecules was formed when the neurotrophin was fused to a carboxyl terminus of the CH3 region of an IgG molecule, as illustrated in Figure 18. The surprising observation of the maintenance of the high affinity binding of BDNF for TrkB (Figure 24), despite fusion to the HIRMAb heavy chain (Figure 18), is consistent with the fact that BDNF normally binds to TrkB as a dimer.

Example 5

[00195]   **Human neural cells subjected to hypoxia are neuroprotected by the fusion protein with equal activity as recombinant BDNF.** Human SH-SY5Y neural cells were exposed to 10 uM retinoic acid for 7 days, which induces gene expression of trkB, the BDNF receptor. The cells were then exposed to 16 hours of oxygen deprivation in a sealed chamber, with oxygen sensor. Excitotoxic neural damage was then induced by 4 hours of re-oxygenation (Figure 25A). During

this 4 hour re-oxygenation period, the cells were exposed to either no treatment or equi-molar concentrations of human recombinant BDNF or fusion protein. As shown in Figure 25B, the fusion protein was equipotent with native human BDNF with respect to inducing neuroprotection in human neural cells exposed to excitoxic ischemia-re-oxygenation.

Example 6

[00196]   **High affinity binding of fusion protein to human blood-brain barrier insulin receptor in isolated human brain capillaries.** Isolated human brain capillaries are used as an in vitro model system of the human BBB (Figure 26A). The fusion protein was radiolabeled with 3H-N-succinimidyl propionate, and added to the human brain capillaries to establish a radio-receptor assay (RRA) of fusion protein binding to the HIR of the human BBB. [^{3}H]-fusion protein is specifically bound to the BBB, as the binding is self-inhibited by unlabeled fusion protein (Figure 26B). The fusion protein is bound by the insulin receptor of the human BBB, because the murine HIRMAb (mHIRMAb) also inhibits binding of [^{3}H]-fusion protein to the human BBB. The binding data in Figure 26B were fit to a Scatchard plot with a non-linear regression analysis to produce the binding constants: K_D= 0.55 ± 0.07 nM, B_{max} = 1.35 + 0.10 pmol/mg$_p$, and NSB = 0.39 ± 0.02 pmol/mg$_p$, where K_D is the dissociation constant, Bmax is the maximal binding, and NSB is the non-saturable binding. The KD is <1 nM, which indicate the fusion protein binds the HIR on the human BBB with very high affinity.

Example 7

[00197]   **Pharmacokinetics and brain uptake of fusion protein by the adult Rhesus monkey.** The fusion protein was tritiated with [^{3}H]-N-succinimidyl propionate to a specific activity of 2.0 μCi/μg. A 5 year old female Rhesus monkey, weighing 5.2 kg, was administered by a single intravenous injection a dose of 746 μCi (373 μg), and serum was collected at multiple time points over a 180 min period. The serum glucose of the anesthetized, overnight-fasted primate was constant throughout the 180 min study period, and averaged 72 ± 2 mg%, which indicates that the administration of the HIRMAb based fusion protein caused no interference of the endogenous insulin receptor, and had no effect on glycemia control.

[00198]   The serum removed from the anesthetized Rhesus monkey was analyzed for total radioactivity (Figure 27A), and radioactivity that was precipitable by trichloroacetic acid (TCA) (Figure 27B). At 180 minutes after drug injection, the animal was euthanized, and brain radioactivity was analyzed with the capillary depletion method (Figure 27C), similar to prior work on the brain uptake of [^{125}I]-labeled murine HIRMAb in the Rhesus monkey. Based on the specific activity of the [^{3}H]-fusion protein, the brain radioactivity was converted to ng per gram (g) brain, as shown in Figure 27D, and this level was compared to the reported endogenous concentration of BDNF in the adult primate brain.

[00199]   The plasma pharmacokinetics analysis (Figure 27A) shows that the fusion protein of the
genetically engineered HIRMAb and the BDNF is removed from blood at the same rate as the
original murine HIRMAb. This is an important finding, because it shows that the fusion of BDNF,
a highly cationic protein, to the HIRMAb does not accelerate the blood clearance of the HIRMAb.
Prior work shows that the attachment of the cationic BDNF to a monoclonal antibody greatly
accelerates the blood clearance of the antibody, owing to the cationic nature of the BDNF, which
greatly enhances hepatic uptake. The work in Figure 27A shows that when the cationic BDNF was
re-engineered as an IgG fusion protein, the plasma pharmacokinetics was dominated by the IgG
moiety, and that the blood level of the BDNF remains high for a prolonged period.

[00200]   The data in Figure 27B show that when BDNF was re-formulated as an IgG fusion protein,
the metabolic stability of the neurotrophin in blood was greatly enhanced, as compared to the native
BDNF. Owing to its cationic nature, the native BDNF was rapidly removed from blood, and was
rapidly degraded into TCA-soluble radioactive metabolites (Figure 27B). However, the TCA-
insoluble form of the labeled fusion protein remains high during the 3 hours after an intravenous
injection in the primate (Figure 27B). The data in Figures 27A,B show the advantages of re-
engineering a neurotrophin pharmaceutical as a fusion protein. The native neurotrophin was rapidly
removed from blood and was rapidly degraded. However, the plasma pharmacokinetics profile, and
metabolic stability profile, of the neurotrophin resemble those of an IgG molecule, when the IgG-
neurotrophin fusion protein was produced.

[00201]   Native BDNF is not transported across the BBB. Similarly, a [^{3}H]-mouse IgG2a isotype
control antibody was not transported across the BBB in the adult Rhesus monkey, as the brain
volume of distribution (V_D) of the IgG at 180 minutes after an intravenous injection was equal to
the plasma volume, 18 μL/g (Figure 27C, open bars). Conversely, the brain V_D of the [^{3}H]-fusion
protein exceeds 140 μl/g brain (Figure 27C, closed bars). Capillary depletion analysis separates the
brain vasculature from the post-vascular supernatant, and allows detection of the transport of a drug
through the BBB and into brain, as opposed to simple sequestration of the drug by the brain
vasculature. The brain V_D of the post-vascular supernatant of the [^{3}H]-fusion protein was equal to
the V_D of the brain homogenate (Figure 27C), which indicates the fusion protein was transported
through the BBB and into brain parenchyma.

[00202]   The brain V_D of the fusion protein was converted into ng fusion protein per gram brain,
based on the specific activity of the [^{3}H]-fusion protein, and this allowed for calculation of the total
mass of fusion protein in the brain, 24 $\pm$ 1 ng/g, as shown in Figure 27D. This value is >10-fold
higher than the endogenous brain concentration of BDNF in the adult primate (45). Therefore, the
administration of a dose of 373 μg to a 5.2 kg Rhesus monkey, which is equal to a normalized dose
of 72 μg/kg of fusion protein, results in a marked increase in the brain concentration of BDNF.
Such an increase in brain BDNF, following intravenous administration, is not possible with native
BDNF, because the native BDNF does not cross the BBB. However, when BDNF is re-engineered

in the form of the fusion protein, then pharmacologically active levels of the neurotrophin in brain are achieved (Figure 27D).

[00203]   The data shows that: (1) the plasma mean residence time (MRT) of the fusion protein, 312 minutes, was 100-fold greater than the MRT for native BDNF, which was 3.0 minutes, and (2) the systemic clearance of the fusion protein, 0.94 mL/min/kg, was 39-fold slower than the systemic clearance of the BDNF, which was 37 mL/min/kg. In other words, the average blood level of the recombinant protein was up to 100-fold greater when the recombinant protein was re-formulated as an IgG fusion protein. Thus, fusion of the BDNF to the molecular Trojan horse had 2 benefits: (1) the molecular Trojan horse carried the BDNF across the blood-brain barrier (BBB), whereas the BDNF alone cannot cross the BBB, and (2) the molecular Trojan horse prevented the rapid loss from blood of the neurotrophin; BDNF by itself lasts only about 3 minutes in the blood. Both of these properties serve to enhance the pharmacological effect of the BDNF in brain following administration into the blood stream. See, e.g., Table 5.

Table 5. Pharmacokinetic parameters for [3H]-fusion protein and [3H]-BDNF

Parameter [3H]-BDNF	[3H]-fusion protein	
A_1 (%ID/ml)	0.147 ± 0.020	5.28 ± 0.60
A_2 (%ID/ml)	0.061 ± 0.005	2.26 ± 0.32
k_1 (min^{-1})	0.195 ± 0.050	1.75 ± 0.26
k_2 (hr^{-1})	0.186 ± 0.042	15.6 ± 0.6
$t_{1/2}^{1}$ (min)	3.5 ± 0.9	0.42 ± 0.07
$t_{1/2}^{2}$ (hr)	3.7 ± 0.9	0.045 ± 0.001
CL_{SS} (ml/min/kg)	0.94 ± 0.16	37.0 ± 2.5
MRT (min)	312 ± 78	3.0 ± 0.3

A_1, A_2, k_1, and k_2 are the intercepts and slopes of the bi-exponential function describing the decay in plasma concentration with time. The parameters for the fusion protein were determined for the Rhesus monkey, and the parameters for BDNF were determined in the adult rat. All data are normalized for differences in body weight.

$t_{1/2}^{1}$ and $t_{1/2}^{2}$ are computed from k_1 and k_2, respectively, and are the half-times of the decay curves for each exponent.

CL_{ss} and MRT are the steady state clearance and mean residence time, respectively, and are computed from A_1, A_2, k_1, and k_2 using standard pharmacokinetic formulations.

Example 8

[00204]   **Neuroprotection in regional brain ischemia by conjugates of BDNF and a BBB molecular Trojan horse.** Numerous attempts have been made to develop neuroprotective agents for the treatment of acute stroke. There have been no successes to date because the neuroprotective drugs are either too toxic, in the case of certain small molecules, or ineffective, because the drug does not cross the BBB. BDNF is neuroprotective when injected directly in the brain in parallel with experimental stroke in rodents and regional brain ischemia. The BDNF must be injected across the skull bone into the brain, because this large molecule drug does not cross the BBB. Since the BBB is intact in the early hours after regional brain ischemia, and since BDNF does not cross the BBB, then there is no neuroprotective effect in the ischemic brain following the intravenous administration of BDNF alone. To deliver BDNF across the BBB, the neurotrophin was attached to a mouse MAb to the rat transferrin receptor (TfR). This peptidomimetic MAb carries BDNF across the BBB, and the combined BDNF-MAb conjugate is highly neuroprotective following delayed intravenous administration in experimental stroke, because the BDNF is able to cross the BBB and enter the brain from blood. Once inside the brain, and behind the BBB, the BDNF activates its cognate receptor, trkB, which then induces neuroprotection in ischemic neurons, and stops the apoptotic death cycle. The neuroprotective effect of the BDNF-MAb conjugate demonstrates a dose response effect, a time response effect, and is long-lasting, as the neuroprotection at 7 days is identical to the neuroprotection at 1 day after a single intravenous administration of the BDNF-MAb conjugate. See, e.g., Zhang and Pardridge (2001) Brain Res. 889:49-56, and Zhang and Pardridge (2001) Stroke 32:1378-1374, which are incorporated by reference herein in their entirety. The fusion protein would also be neuroprotective in human stroke, since the BDNF is fused to an MAb to the HIR, which rapidly binds to both the human BBB in vitro, and is rapidly transported across the primate BBB in vivo.

Example 9

[00205]   **Neuroprotection in global brain ischemia of conjugates of BDNF and a BBB molecular Trojan horse.** The direct injection of BDNF into the brain is also neuroprotective in transient forebrain ischemia (TFI), such as might occur after a cardiac arrest. However, intravenous BDNF is not neuroprotective in TFI, because the BDNF does not cross the BBB, and because the BBB is intact in the early hours after TFI, when neuroprotection is still possible. Conversely, intravenous BDNF was neuroprotective in TFI if the BDNF was attached to a mouse MAb against the rat transferrin receptor (TfR), which acts as a molecular Trojan horse to ferry the BDNF across the BBB and into brain. Adult rats were subjected to TFI, which resulted in a flat-line electroencephalogram (EEG) for approximately a 10-minute period. The animals were resuscitated

and then administered 1 of 4 different therapeutics intravenously: (a) buffer, (b) unconjugated BDNF, (c) the receptor specific MAb without the BDNF attached, and (d) the BDNF-MAb conjugate. In the case of the animals treated with saline, unconjugated BDNF, or MAb alone, there was no neuroprotection of pyramidal neurons in the CA1 sector of hippocampus. However, in the case of the BDNF-MAb conjugate, there is complete normalization of CA1 pyramidal neuron density following delayed intravenous administration. See, e.g., Wu and Pardridge (199), PNAS (USA) 96:254-259, which is incorporated by reference herein in its entirety. This shows that BDNF is strongly neuroprotective in global brain ischemia following delayed intravenous administration, providing the BDNF is attached to a BBB molecular Trojan horse. The recombinant fusion protein of BDNF and a receptor specific MAb could be given following cardiac arrest to prevent permanent brain damage.

Example 10

[00206]   **BDNF is neuroprotective in brain and spinal cord injury if the neurotrophin can access brain cells.** BDNF is neuroprotective in brain injury, providing the neurotrophin is injected directly through the skull bone, because BDNF does not cross the BBB. BDNF is also neuroprotective in brain subjected to excitotoxic injury by neurotoxins, and is neuroprotective in brain infected with the human immune deficiency virus (HIV)-1. BDNF is also neuroprotective in acute spinal cord injury; however, the BDNF must be administered by direct infusion into the spinal canal, because the BDNF does not cross the blood-spinal cord barrier, which is the same as the BBB in the forebrain. In all these cases, the intravenous administration of BDNF would not be neuroprotective, because the BDNF does not cross the BBB, and the BBB is intact in brain injury in the early hours after the injury, when neuroprotection is still possible. Conversely, the BDNF fusion protein would be neuroprotective in these conditions following intravenous administration, because the BDNF is fused to the BBB molecular Trojan horse, and is able to penetrate the brain and spinal cord from the blood following peripheral administration.

Example 11

[00207]   **BDNF is neuroprotective in chronic neurodegenerative conditions of brain if the neurotrophin can access brain cells.** Neurotrophins, such as BDNF can be developed as drugs for peripheral routes of administration, providing the neurotrophin is enabled to cross the BBB. Fusion of BDNF to the chimeric HIRMAb offers a new approach to the non-invasive delivery of BDNF to the brain in humans for the chronic treatment of neurodegenerative disease, including prion diseases, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), ALS, transverse myelitis, motor neuron disease, Pick's disease, tuberous sclerosis, lysosomal storage disorders, Canavan's disease, Rett's syndrome, spinocerebellar ataxias, Friedreich's ataxia, optic atrophy, and retinal degeneration, and brain aging.

Example 12

[00208]   **BDNF as a therapeutic in retinal degeneration and blindness.** The retina, like the

brain, is protected from the blood by the blood-retinal barrier (BRB). The insulin receptor is

expressed on both the BBB and the BRB, and the HIRMAb has been shown to deliver therapeutics

to the retina via RMT across the BRB (Zhang et al, (2003) Mol. Ther. 7: 11-18). BDNF is

neuroprotective in retinal degeneration, but it was necessary to inject the neurotrophin directly into

the eyeball, because BDNF does not cross the BRB. The fusion protein could be used to treat

retinal degeneration and blindness with a route of administration no more invasive than an

intravenous or subcutaneous injection, because the HIRMAb would deliver the BDNF across the

BRB, so that the neurotrophin would be exposed to retinal neural cells from the blood

compartment.

Example 13

[00209]   **BDNF as a therapeutic for depression.** A subset of patients with depression may have a

brain deficiency of BDNF, and the correlation of single nucleotide polymorphisms (SNPs) with

affective disorders has been reported. The direct injection of BDNF into the brain has durable anti-

depressant effects in rodent model. The BDNF must be injected directly into the brain, because the

neurotrophin does not cross the BBB. The chronic administration of the fusion protein would

provide a means for elevating the brain levels of BDNF, and may be therapeutic in those patients

with depression and a reduced production of brain BDNF.

Example 14

[00210]   **Method of manufacturing IgG fusion proteins.** The transfection of a eukaryotic cell

line with immunoglobulin G (IgG) genes generally involves the co-transfection of the cell line with

separate plasmids encoding the heavy chain (HC) and the light chain (LC) comprising the IgG. In

the case of a IgG fusion protein, the gene encoding the recombinant therapeutic protein may be

fused to either the HC or LC gene. However, this co-transfection approach makes it difficult to

select a cell line that has equally high integration of both the HC and LC-fusion genes, or the HC-

fusion and LC genes. The preferred approach to manufacturing the fusion protein is the production

of a cell line that is transfected with a single plasmid DNA that contains all the required genes on a

single strand of DNA, including the HC-fusion protein gene, the LC gene, the selection gene, e.g.

neo, and the amplification gene, e.g. the dihydrofolate reductase gene. As shown in the diagram of

the fusion protein tandem vector in Figure 12, the HC-fusion gene, the LC gene, the neo gene, and

the DHFR gene are all under the control of separate, but tandem promoters and separate but tandem

transcription termination sequences. Therefore, all genes are equally integrated into the host cell

genome, including the fusion gene of the therapeutic protein and either the HC or LC IgG gene.

Example 15

[00211]   **Construction and Use of GDNF-immunoglobulin fusion proteins.** Glial-derived
neurotrophic factor (GDNF) is a potent neuroprotective neurotrophin (Lin *et al, Science* 260:1130-
1132 (1993)). GDNF could be developed as a neuroprotective drug for acute conditions, such as
acute ischemic stroke, or chronic conditions, such as Parkinson's disease, or motor neuron disease,
or post-stroke neural repair (Lapchak *et al, Neuroscience* 78:61-72 (1997); Kitagawa *et al*, Stroke
29:1417-1422 (1998); Bohn, *Exp. Neurol.* 190:263-275 (2004); Kobayashi, *et al. Stroke* 37:2361-
2367 (2006)). However, GDNF, like other large molecule drugs, does not cross the blood-brain
barrier (BBB) (Kastin *et al, Neurosci Lett.* 340:239-241(2003)). Owing to the BBB problem, prior
human clinical trials with recombinant GDNF delivered the protein to brain via invasive trans-
cranial routes, such as intra-cerebroventricular injection (Lang *et al, Ann Neurol.* 59:459-466
(2006)) or convention enhanced diffusion (Patel *et al, Ann. Neurol.* 57:298-302 (2005)). However,
both approaches were limited by poor GDNF penetration into the brain, as well as adverse events
related to the invasive drug delivery system.

[00212]   Large molecule drugs, such as GDNF, could be delivered to brain non-invasively, via the
trans-vascular route across the BBB with the use of molecular Trojan horse technology (Pardridge,
Pharm. Res. 27:1733-1744 (2007)). A BBB molecular Trojan horse is an endogenous peptide, or
receptor-specific peptidomimetic monoclonal antibody (MAb) that undergoes receptor-mediated
transport across the BBB via endogenous peptide receptors, such as the transferrin receptor or the
insulin receptor. Prior work has developed genetically engineered chimeric or humanized MAb's to
the human insulin receptor (HIR), as molecular Trojan horses for the human brain (Boado *et al,
Biotechnol. Bioeng.* 96:381-391 (2007)). The non-transportable protein therapeutic is fused to the
heavy chain (HC) of the HIRMAb. It is essential that the genetically engineered fusion protein
retain bi-functionality, and both bind with high affinity to the HIR, to cause BBB transport, and
bind to the cognate receptor on brain cells, to induce the desired pharmacologic effect, once the
fusion protein penetrates the brain.

[00213]   The present studies described the genetic engineering of a fusion protein of the chimeric
HIRMAb and human GDNF. The amino terminus of mature human GDNF is fused to the carboxyl
terminus of the HC of the HIRMAb (Figure 28). Following expression in COS cells, the bi-
functionality of the HIRMAb-GDNF fusion protein is evaluated with receptor binding assays for
both the HIR and the human GDNF receptor (GFR)-α1. The GDNF biologic activity of the fusion
protein is also evaluated with bio-assays using 2 human neural cell lines, the SH-SY5Y line, and the
SK-N-MC line. Finally, the in vivo neuroprotective effects of the HIRMAb-GDNF fusion protein
is demonstrated in rat brain using the middle cerebral artery occlusion model (MCAO) of acute
ischemic stroke.

MATERIALS AND METHODS

Cloning of GDNF cDNA

[00214]   The human prepro GDNF cDNA (GenBank P39905) corresponding to amino acids Met⁻¬1-Ile211 was cloned by the polymerase chain reaction (PCR) using the oligodexoynucleotides (ODNs) described in Table 6 and cDNA derived from reverse transcription of polyA+RNA isolated from human U87 glial cells. DNA sequence analysis was performed and the nucleic acid and amino acid sequences of the cloned human prepro GDNF are given in SEQ ID NOs:43 and 44, respectively.

Table 6. Oligodeoxynucleotide primers used in the RT-PCR cloning of human GDNF and in the engineering of the HIRMAb-GDNF expression vector

Human prepro GDNF FWD: phosphate-ATGAAGTTATGGGATGTCGTGGCTG (**SEQ ID NO:40**)

Human prepro GDNF REV: phosphate-TCAGATACATCCACACCTTTTAGCG (**SEQ ID NO:41**)

Mature human GDNF FWD: phosphate-CATCACCAGATAAACAAATGGCAGTG (**SEQ ID NO:42**)

[00215]   The GDNF cDNA was cloned by PCR using 25 ng polyA+RNA-derived cDNA, 0.2 µM forward and reverse ODN primers (Table 6), 0.2 mM deoxynucleosidetriphosphates, and 2.5 U PfuUltra DNA polymerase (Stratagene, San Diego, CA) in a 50 µl Pfu buffer (Stratagene). The amplification was performed in a Mastercycler temperature cycler (Eppendorf, Hamburg, Germany) with an initial denaturing step of 95°C for 2 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec and amplification at 72°C for 1 min; followed by a final incubation at 72°C for 10 min. PCR products were resolved in 0.8% agarose gel electrophoresis, and the expected major single band of ~0.6 kb corresponding to the human GDNF cDNA was produced (Figure 29A). The human prepro GDNF cDNA was subcloned into the pcDNA3.1 eukaryotic expression plasmid, and was designated pCD-GDNF. The engineering of this plasmid was validated by DNA sequencing, and by expression of immunoreactive GDNF in COS cells transfected with pCD-GDNF.

Engineering of HIRMAb-GDNF expression vector

[00216]   For the engineering of the pHIRMAb-GDNF heavy chain (HC) expression plasmid, the mature human GDNF cDNA (SEQ ID NO:51) corresponding to amino acids Ser⁻¬78-Ile211 (SEQ ID NO:52) of full length human GDNF was cloned by PCR using the pCD-GDNF as template.

TCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCA
GCTGCCAACCCAGAGAATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAAC
CGGGGTTGTGTCTTAACTGCAATACATTTAAATGTCACTGACTTGGGTCTGGGCTATGA
AACCAAGGAGGAACTGATTTTTAGGTACTGCAGCGGCTCTTGCGATGCAGCTGAGACA

ACGTACGACAAAATATTGAAAAACTTATCCAGAAATAGAAGGCTGGTGAGTGACAAA
GTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGATGATGACCTGTCGTTTTTAGATGA
TAACCTGGTTTACCATATTCTAAGAAAGCATTCCGCTAAAAGGTGTGGATGTATCTGA
(SEQ ID NO:51)

SPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYET
KEELIFRYCSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLSFLDDNLVY
HILRKHSAKRCGCI (SEQ ID NO:52)

[00217]   The PCR cloning reaction was performed as described above. The ODNs used for PCR are
5'-phosphorylated for direct insertion into the HpaI site of the pHIRMAb-HC expression plasmid
(Figure 29B). The pHIRMAb-HC plasmid encodes the HC of the chimeric HIRMAb, and dual
transfection of COS cells with this plasmid and a light chain (LC) expression plasmid, pHIRMAb-
LC, allows for transient expression of either the chimeric HIRMAb, or a fusion protein, in COS
cells. The mature human GDNF forward (FWD) PCR primer (Table 6) introduces "CA"
nucleotides to maintain the open reading frame and to introduce a Ser-Ser linker between the
carboxyl terminus of the CH3 region of the HIRMAb HC and the amino terminus of the GDNF.
The fusion of the GDNF monomer to the carboxyl terminus of each HC is depicted in Figure 28.
This design sterically restricts the GDNF to a dimeric configuration, which replicates the native
dimeric conformation of GDNF that binds to the GFRα1 (Xu et al, 1998; Eketjall et al, 1999). The
GDNF reverse (REV) PCR primer introduces a stop codon, "TGA," immediately after the terminal
isoleucine of the mature GDNF protein, and it is identical to the human prepro GDNF REV ODN
primer (Table 6) used in the cloning of the human prepro GDNF cDNA. The engineered
pHIRMAb-GDNF expression vector was validated by DNA sequencing. The nucleic acid and
amino acid sequences for fusion heavy chain (SEQ ID NOs 45 and 46) are as follow:

ATGGACTGGACCTGGAGGGTGTTCTGCCTGCTTGCAGTGGCCCCCGGAGCCCACAGCCAGGTTCAG
CTGCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTTAGTGAAGATATCCTGCAAGGCTTCT
GGTTACACCTTCACAAACTACGATATACACTGGGTGAAGCAGAGGCCTGGACAGGGACTTGAGTGG
ATTGGATGGATTTATCCTGGAGATGGTAGTACTAAGTACAATGAGAAATTCAAGGGCAAGGCCACA
CTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCACCTCAGCAGCCTGACTTCTGAGAAATCT
GCAGTCTATTTCTGTGCAAGAGAGTGGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA
GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACA
GCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGC
GCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGC
AGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAG
CCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCA
CCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGAC
ACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCT

GAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG

GAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAAT

GGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCC

AAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC

AAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGG

GAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCC

TTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGC

TCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA

AGTTCATCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCAGCT

GCCAACCCAGAGAATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGTTGTGTC

TTAACTGCAATACATTTAAATGTCACTGACTTGGGTCTGGGCTATGAAACCAAGGAGGAACTGATT

TTTAGGTACTGCAGCGGCTCTTGCGATGCAGCTGAGACAACGTACGACAAAATATTGAAAAACTTA

TCCAGAAATAGAAGGCTGGTGAGTGACAAAGTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGAT

GATGACCTGTCGTTTTTAGATGATAACCTGGTTTACCATATTCTAAGAAAGCATTCCGCTAAAAGG

TGTGGATGTATCTGA (SEQ ID NO:45)

MDWTWRVFCLLAVAPGAHSQVQLQQSGPELVKPGALVKISCKASGYTFTNYDIHWVKQRPGQGLEW

IGWIYPGDGSTKYNEKFKGKATLTADKSSSTAYMHLSSLTSEKSAVYFCAREWAYWGQGTLVTVSA

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLS

SVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKD

TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN

GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW

ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SSSPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYETKEELI

FRYCSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLSFLDDNLVYHILRKHSAKR

CGCI (SEQ ID NO:46). Also shown in Figure 36.

[00218]   The HIRMAb HC and LC cDNA expression cassettes are driven by the cytomegalovirus
(CMV) promoter and contain the bovine growth hormone (BGH) polyadenylation (pA) sequence
(Figure 29B). The engineering of the universal pHIRMAb-HC vector was performed by insertion
of a single HpaI site at the end of the HIRMAb HC CH3 open reading frame (orf) by site directed
mutagenesis (SDM).

Transient expression of HIRMAb-GDNF fusion protein in COS cells

[00219]   COS cells were dual transfected with pHIRMAb-LC and pHIRMAb-HC-GDNF using
Lipofectamine 2000, with a ratio of 1:2.5, ug DNA:uL Lipofectamine. Following transfection, the
cells were cultured in serum-free VP-SFM (Invitrogen, Carlsbad, CA). The conditioned serum free
medium was collected at 3 and 7 days. The fusion protein was purified by protein A affinity
chromatography.

Human IgG and GDNF ELISAs

[00220]   Human IgG ELISA was performed in Immulon 2 high binding plates (Dynex Tech., Chantilly, VA) with COS cell conditioned medium. A goat anti-human IgG primary antibody (Zymed-Invitrogen, Carlsbad, CA) was plated in 0.1 M NaHCO3 (100 µl, 2 µg/ml) and incubated for overnight at 4C. Plates were washed 0.01 M Na2HPO4/0.15 M NaCl/pH=7.4/0.05% Tween-20 (PBST), and blocked with 1% gelatin in PBST for 30 min at 22°C. Plates were incubated with 100 µL/well of either human IgG1 standard or the fusion protein for 60 minutes at room temperature (RT). After washing with PBST, a goat anti-human kappa LC antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was plated for 60 min at 37°C. Color development was performed with p-nitrophenyl phosphate (Sigma) at pH=10.4 in the dark. The reaction was stopped with NaOH, and absorbance at 405 nm was measured in a BioRad ELISA plate reader. Human GDNF immunoreactivity in COS cell conditioned medium was measured with the double antibody sandwich GDNF Emax Immunoassay System kit from Promega (Madison, WI).

SDS-PAGE and Western blotting

[00221]   The homogeneity of protein A purified fusion protein produced by COS cells was evaluated with a reducing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomasie Blue staining. For Western blotting, immunoreactivity was tested with a primary rabbit antibody to human GDNF (Santa Cruz Biotechnology, Santa Cruz, CA) or a primary goat antiserum against human IgG heavy and light chains (Vector Labs, Burlingame, CA).

HIR receptor assay

[00222]   The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA using the lectin affinity purified HIR ECD. CHO cells transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column. The HIR ECD (0.2 µg/well) was plated on Immulon 2 high binding 96-well plates, and the binding of the chimeric HIRMAb, the HIRMAb-GDNF fusion protein, or human IgG1 to the HIR ECD was detected with a biotinylated goat anti-human IgG (H+L) antibody (0.3 µg/well), and the ABC Elite detection system (Vector Labs). The concentration that caused 50% binding to the HIR ECD, the ED50, was determined by non-linear regression analysis using the WinNonlin software.

GFRα1 receptor assay

[00223]   Binding of recombinant GDNF (Peprotech, Rocky Hill, NJ), the chimeric HIRMAb, or the HIRMAb-GDNF fusion protein to the ECD of recombinant human GFRα1 was measured with an ELISA. A mouse anti-human (MAH) IgG (Zymed-Invitrogen) was plated in 96-well plates overnight at 2 µg/well. Following aspiration, washing, and blocking with 1% bovine serum albumin (BSA), the Fc fusion of the human GFRα1 ECD (R&D Systems, Minneapolis, MN) was plated at 0.4 µg/well. Following an incubation at room temperature (RT) for 60 min, the wells were washed with PBST, and the GDNF, antibody, or fusion protein was plated for 2 hours at RT.

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Following washing in PBST, a goat anti-GDNF antibody (R&D Systems) was plated at 0.4 ug/well for 30 min at RT. Following washing in PBST, a conjugate of alkaline phosphatase (AP) and a rabbit anti-goat (RAG) IgG(H+L) (Vector Labs) was plated and detection at 405 nm was performed with an ELISA plate reader after color development with para-nitrophenylphosphate (Sigma Chemical Co.).

SK-N-MC bio-assay

[00224]   Human neural SK-N-MC cells were dual transfected with the c-ret kinase and a luciferase reporter plasmid under the influence of the 5'-flanking sequence (FS) of the rat tyrosine hydroxylase (TH) gene. The cells were grown in collagen coated 24-well dishes to 70% confluency in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 400 μg/mL G418. To begin the assay, the medium was aspirated, and 400 μL/well of fresh DMEM/10% FBS was added along with either recombinant GDNF or fusion protein. After a 24 hour incubation at 37°C, the wells were aspirated, and the cells extracted with 200 μL/well of Luciferase Reporter Lysis buffer (Promega, Madison, WI). Following centrifugation, luciferase enzyme activity was measured in the lysate with a luminometer, and luciferase enzyme activity, reported as picogram (pg) of luciferase, was normalized per mg sample protein using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL).

SH-SY5Y bio-assay

[00225]   Human neural SH-SY5Y cells were plated in 96 well plates in DMEM/Ham F12 (1:1) in 10% FBS and grown until the cells reached 70% confluency. The medium was supplemented with 10 μM retinoic acid to induce differentiation of the cells over a 10 day period. After cell differentiation, the medium was changed, and supplemented with either GDNF or HIRMAb-GDNF fusion protein at zero time, and cell proliferation was measured over the 5 days with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega), which uses the tetrazolium compound, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS), and the electron coupling reagent, phenazine methosulfate (PMS). Absorbance at 490 nm is a measure of formazan production, which is proportional to the number of viable cells.

Middle cerebral artery occlusion model

[00226]   The permanent middle cerebral artery occlusion (MCAO) model was performed as described previously in adult Sprague-Dawley rats (Zhang and Pardridge, *Brain Res.* 889:49-56 (2001)). The right middle cerebral artery of the anesthetized rat was occluded by insertion of an intra-luminal 4-0 nylon suture; the suture was coated in 0.1% poly-L-lysine and dried at 60°C for 1 hour. Within 15 minutes of the occlusion, the rat was treated with an intra-cerebral injection of either HIRMAb-GDNF fusion protein (130 μg in 10 μL) or an equal volume of saline. Since the fusion protein is 17% GDNF, the dose of fusion protein is equivalent to a dose of GDNF of 22 μg, which is a dose that causes pharmacological effects in the brain (Sullivan et al, 1998). The drug was injected into the brain under stereotaxic guidance with the following coordinates: 0.2 mm posterior

to bregma, 4.0 mm lateral to the midline, and 5.0 mm deep from the dural surface. The arterial
nylon suture was left in place for permanent occlusion of the middle cerebral artery. A total of 20
rats were used for the study; 9 rats (5 treated with saline and 4 treated with HIRMAb-GDNF)
expired prematurely and were excluded from the study. The rats were allowed to recover, and were
euthanized 24 hours later, and coronal sections of brain were prepared with a rat brain matrix (ASI
Instruments, Warren, MI), followed by staining with 2% 2,3,5-triphenyltetrazolium chloride (TTC),
as described previously (Zhang and Pardridge, *Brain Res.* 889:49-56 (2001)).TTC stains healthy
brain red, and infarcted brain is colorless. The stained brain sections were scanned on a UMAX
PowerLook II flatbed scanner, and the area of the hemispheric infarct volumes was determined with
the NIH Image software. The volume of the infarct was computed from the area of the infarcted
zone and length (2 mm) of the coronal slices of brain hemisphere (Zhang and Pardridge, *Brain Res.*
889:49-56 (2001)). The scanned color image was converted to an inverted grayscale image in
Photoshop, so that infarcted brain appears black and healthy brain appears white.

[00227]   The neurologic deficit was determined 24 hours after the occlusion, as described
previously (Zhang and Pardridge, *Stroke* 32:1378-1384 (2001)), and scored as follows: 0, no deficit;
1, failure to extend contralateral forepaw fully; 2, decreased grip of contralateral forelimb while tail
is pulled; 3, spontaneous circling to left; 4, walks only when stimulated with decreased level of
consciousness.

RESULTS

[00228]   DNA sequencing of the expression cassette of the pCD-GDNF encompassed 1,752
nucleotides (nt), including a 715 nt CMV promoter, a 636 nt prepro GDNF open reading frame, and
a 401 nt BGH sequence, which predicted a 211 amino acid human preproGDNF protein, including
a 19 amino acid signal peptide with 100% identity with the known sequence for human GDNF
(P39905). Transfection of COS cells with pCD-GDNF resulted in high levels of immunoreactive
GDNF the medium at 3 and 7 days following transfection.

[00229]   The cDNA corresponding to the 134 amino acid mature GDNF was amplified by PCR
using the ODNs in Table 6 and the pCD-GDNF as template, and this cDNA was subcloned into the
HpaI site of the pHIRMAb-HC plasmid, as outlined in Figure 29B. DNA sequencing of the
expression cassette of the pHIRMAb-GDNF plasmid encompassed 2,890 nt, including a 714 nt
CMV promoter, a 9 nt full Kozak site (GCCGCCACC), a 1,797 nt HIRMAb HC-GDNF fusion
protein open reading frame, and a 370 nt BGH sequence. The plasmid encoded for a 598 amino
acid protein, comprised of a 19 amino acid IgG signal peptide, the 443 amino acid HIRMAb HC, a
2 amino acid linker (Ser-Ser), and the 134 amino acid human GDNF minus the signal peptide or
propeptide. The predicted molecular weight of the heavy chain fusion protein, minus glycosylation,
is 63,860 Da, with a predicted isoelectric point (pI) of 9.03.

[00230]   Dual transfection of COS cells with the pHIRMAb-GDNF and the pHIRMAb-LC resulted
in medium human IgG levels of 2.0 μg/mL, as determined with a human Fc specific ELISA. The
level of immunoreactive GDNF in the medium was measured with a GDNF-specific ELISA. The

data from both the human IgG and human GDNF ELISAs are given in Table 7, and the medium

levels are expressed as nM concentrations to allow for comparison of the 2 assays.

Table 7. HIRMAb-GDNF fusion protein concentrations in the medium of transfected COS cells
following transfection with pHIRMAb-LC and pHIRMAb-GDNF

Target of primary antibody	3 day concentration	7 day concentration
	(nM)	(nM)
Anti-human IgG Fc	3.1 ± 0.2	11.3 ± 1.4
Anti-human GDNF	2.0 ± 0.5	12.6 ± 3.1

Mean ± SE (n=3 dishes per point). The medium immunoreactive human IgG or GDNF
was <0.1 nM in dishes treated with Lipofectamine 2000 alone.

[00231]   As shown in Table 7, the concentration of the HIRMAb-GDNF fusion protein the medium
was the same irrespective of whether the human IgG or GDNF ELISA is used.

[00232]   The HIRMAb-GDNF fusion protein was purified by protein A affinity chromatography.
Following SDS-PAGE and Coomasie blue staining, the size of the light chain (LC) was the same
for both the HIRMAb and the HIRMAb-GDNF fusion protein (Figure 30). The size of the heavy
chain (HC) of the fusion protein was about 15 kDa larger than the HC of the HIRMAb (Figure 30).
On Western blotting, the LC of either the HIRMAb or the HIRMAb-GDNF fusion protein reacted
equally on the Western with a primary antibody directed against the human IgG (H+L), as shown in
Figure 31. The size of the HC of the fusion protein was about 15 kDa larger than the size of the HC
of the HIRMAb on both Western blots using either the anti-human IgG primary antibody (Figure
31) or the anti-human GDNF primary antibody (Figure 31). The anti-GDNF primary antibody
reacts with the HC of the fusion protein, and with recombinant GDNF, but does not react with the
HIRMAb (Figure 31).

[00233]   The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined
with a ligand binding assay using lectin affinity purified HIR ECD (Methods). There is comparable
binding of either the chimeric HIRMAb or the HIRMAb-GDNF fusion protein for the HIR ECD
with ED50 of 0.50±0.11 nM and 0.87±0.14 nM, respectively (Figure 32).

[00234]   The affinity of either GDNF or the HIRMAb-GDNF fusion protein for the ECD of the
human GFRα1 was measured with an ELISA, which is outlined in Figure 33A. Human GDNF
bound to the GFRα1 with an ED50 of 1.03±0.18 nM (Figure 33B, top panel). The affinity of the
HIRMAb-GDNF fusion protein for the GFRα1 was also high, with an ED50 of 1.68±0.17 nM
(Figure 33B, bottom panel).   The biologic activity of GDNF or the HIRMAb-GDNF fusion protein
was also evaluated with bio-assays in human neural cell lines. The human neural SK-N-MC cell
line was dual transfected with the c-ret kinase, and with a luciferase expression plasmid under the

influence of the 5'-flanking sequence (FS) of the rat TH promoter. Addition of GDNF to the
medium activates the TH promoter via activation of the GFRα1/c-ret kinase system, and this leads
to increased luciferase expression (Figure 34A). GDNF increased luciferase expression with an
ED50 of 1.03±0.31 nM, and the HIRMAb-GDNF fusion protein also increased luciferase
expression with an ED50 of 1.68±0.45 nM (Figure 34B). Both GDNF and the HIRMAb-GDNF
fusion protein activated cell division of human neural SH-SY5Y cells following retinoic acid
differentiation, and both proteins increased cell division about 50% over a 5 day incubation
incubation period (Table 8).

Table 8. Enhanced proliferation of retinoic acid differentiated human neural SH-SY5Y cells by
human GDNF or HIRMAb-GDNF

Treatment	A490
GDNF (3 nM)	0.43 ± 0.02 [a]
HIRMAb-GNDF (5 nM)	0.42 ± 0.03 [a]
control	0.29 ± 0.01

Mean ± SE (n=6); a: p<0.005. Cell proliferation measured with the MTS assay as described herein.

[00235]   The intra-cerebral injection of the HIRMAb-GDNF fusion protein caused a 77% reduction
in hemispheric stroke volume, from 331 ± 33 mm3 to 77 ± 22 mm3 (mean ± SE, n=5-6 rats per
group), which was significant at the p<0.001 level (Student's t-test), as shown in Figure 35A.
Similarly, the cortical stroke volume was reduced 86% from 241 ± 26 mm3 to 34 ± 9 mm3
(p<0.001), and the sub-cortical stroke volume was reduced 52% from 90 ± 10 mm3 to 43 ± 16 mm3
(p<0.025), as shown in Figure 35A. A representative TTC stain of the brain 24 hours after
permanent MCAO and intra-cerebral injection of either saline or the HIRMAb-GDNF fusion
protein is shown in Figures 35B and 35C, respectively. The neurologic deficit score was 3.0 ± 0.7
and 1.3 ± 0.6 (mean ± SE, n=5-6 rats per group) in the saline treated and HIRMAb-GDNF treated
rats, respectively, and this improvement in neurologic deficit was significant at the p<0.05 level
(Student's t-test).

DISCUSSION

[00236]   The results of this study were consistent with the following conclusions. First, a bi-
functional IgG-GDNF fusion protein was genetically engineered, wherein mature human GDNF
was fused to the carboxyl terminus of the heavy chain (HC) of a chimeric HIRMAb (Figure 28),
and expressed and secreted in COS cells (Table 8, Figures 30 and 31). Second, the HIRMAb-GDNF
fusion protein was bi-functional and bound the HIR and human GFRα1 with high affinity (Figures
32 and 33). Third, the HIRMAb-GDNF fusion protein had activity in bio-assays of human neural
cells comparable to recombinant human GDNF (Figure 34, Table 8). Fourth, the HIRMAb-GDNF

was neuroprotective in rat brain in vivo in the permanent MCAO stroke model, and caused a marked reduction in stroke volume (Figure 35).

[00237]   GDNF is a member of the transforming growth factor (TGF)-α gene family, along with other neurotrophins, such as neuturin, persephin, or artemin. GDNF reacts with its cognate receptor, GFRαI, as a homo-dimer (Eketjall et al, 1999). Therefore, the GDNF fusion construct described in this work (Figure 28) places GDNF in a dimeric configuration that mimicked the conformation of the neurotrophin at the receptor. The prepro GDNF could be fused to the amino terminus of the HIRMAb HC. However, this would interfere with HIRMAb binding to the HIR, which would impair transport of the fusion protein across the BBB via the insulin receptor. Moreover, the mature GDNF protein folded into a biologically active conformation, despite the absence of the prepro GDNF peptide in the fusion protein described in this work. The goal of fusion protein engineering was to retain the bi-functional characteristics of the fusion construct, and this was accomplished in the case of the HIRMAb-GDNF fusion protein.

[00238]   The fusion protein was secreted in high amounts to the medium by COS cells co-transfected with the HC expression plasmid, pHIRMAb-GDNF (Figure 29B), and the LC expression plasmid, pHIRMAb-LC (Table 7). The HC of the fusion protein is about 15 kDa larger than the HC of the chimeric HIRMAb, based on either SDS-PAGE gels with Coomassie blue staining (Figure 30), or on Western blot analysis using primary antibodies to either human IgG or human GDNF (Figure 31). The anti-human IgG antibody reacts equally with the LC of the fusion protein or the chimeric HIRMAb, since both proteins use the same LC. Both the HIRMAb-GDNF fusion protein and the HIRMAb bind with high affinity to the HIR, and with comparable affinity (Figure 32).

[00239]   The HIRMAb-GDNF fusion protein retains high affinity binding to the human GFRα1 receptor, and the affinity constant of binding is comparable to that of recombinant GDNF (Figure 33B). The high affinity binding of the HIRMAb-GDNF fusion protein to the GFRα1 was translated into biological activity in two different human neural cell lines. The SH-SH5Y cell line expresses the GFRα11, but does not express the c-ret kinase in the undifferentiated state (Xiao *et al, J. Neurochem.* 82:701-808 (2002)). Both the GFRα1 and the c-ret kinase must be expressed to enable GDNF activation of a neural cell (Cik et al, 2000). However, differentiation of SH-SH5Y cells by retinoic acid causes an induction of the expression of the c-ret kinase (Xiao *et al, J. Neurochem.* 82:701-808 (2002)). Following differentiation by retinoic acid, the SH-SY5Y cells demonstrate enhanced proliferation in response to either recombinant human GDNF or the HIRMAb-GDNF fusion protein (Table 8). The SK-N-MC human neural cell line also expresses the GFRα1 receptor, but not the c-ret kinase (Hirata and Kiuchi, *Brain Res.* 983:1-12 (2003)). However, following co-transfection of these neural cells with a c-ret kinase cDNA and a plasmid encoding luciferase under the influence of 2 kb of the 5'-FS of the rat TH promoter, these cells respond to GDNF (Tanaka *et al, Brain res. Brain Res. Protoc.* 11:119-122 (2003)). As outlined in Figure 34A, the extracellular GDNF binds the GFRα1, which activates the c-ret kinase. which

induces a signal transduction cascade leading to activation of the TH promoter. Accordingly, both recombinant GDNF and the HIRMAb-GDNF fusion protein caused an approximate 500% increase in cellular luciferase enzyme activity. The GDNF ED50 in the luciferase assay is 1.0 ± 0.1 nM, and the HIRMAb-GDNF ED50 in this assay is 1.7 ± 0.5 nM (Figure 34B). These ED50 values are identical to the ED50 of saturable binding of either GDNF or the HIRMAb-GDNF fusion protein in the GFRα1 binding assay (Figure 33B). This correlation indicates that the rate-limiting step in activation of neural pathways by GDNF is binding to the cell membrane GFRα1 receptor.

[00240]   The intra-cerebral injection of the HIRMAb-GDNF fusion protein in rat brain following permanent middle cerebral artery occlusion (MCAO) resulted in a 77% reduction in hemispheric stroke volume from 331 ± 33 mm^3 to 77 ± 22 mm3 (p<0.001), as shown in Figure 35A. The neuroprotection was both cortical and sub-cortical, as the cortical stroke volume was reduced 86% from 241 ± 26 mm^3 to 34 ± 9 mm3 (p<0.001), and the sub-cortical stroke volume is reduced 52% from 90 ± 10 mm3 to 43 ± 16 mm3 (p<0.025) (Figure 35A). A representative TTC stain of the rat brain at 24 hours after the permanent MCAO is shown in Figure 35B, for the saline treated rat, and in Figure 35C for the HIRMAb-GDNF fusion protein treated rat. The reduction in stroke volume was correlated with a functional improvement as the neurologic deficit was reduced from 3.0 ± 0.3 to 1.3 ± 0.6 (p<0.05) in the rats treated with the HIRMAb-GDNF fusion protein as compared to the saline treated rats. These findings of *in vivo* neuroprotection of the HIRMAb-GDNF fusion protein correlated with the tissue culture bio-assays (Table 8, Figure 34B), and confirmed the neuroprotective effects of the HIRMAb-GDNF fusion protein on neural cells in brain. The MCAO findings confirmed prior work showing the beneficial effects of intra-cerebral GDNF in acute stroke (Kitagawa et al, 1998). Recent work showed that chronic treatment of the brain with intra-cerebral GDNF may promote striatal neurogenesis following stroke (Kobayashi *et al*, Stroke 37:2361-2367 (2007)). Irrespective of whether the brain is treated acutely or chronically with GDNF, the neurotrophin had to be re-engineered to cross the BBB before clinical trials in human stroke can be initiated.

[00241]   The HIRMAb-GDNF fusion protein could not be delivered to rat brain in the MCAO model following intravenous administration, because the HIRMAb part of the fusion protein is not reactive with the rodent insulin receptor. However, the HIRMAb is active in Old World primates, such as the Rhesus monkey (Pardridge *et al*, *Pharm. Res.* 12:807-816 (1995)). Recent work has shown that fusion proteins of the HIRMAb and brain-derived neurotrophic factor (Boado *et al*, *Biotechnol. Bioeng.* 97:1376-1386 (2007)), a single chain Fv antibody (Boado *et al*, *Bioconjug Chem.* 18:447-455 (2007)), or iduronidase, a lysosomal enzyme (Boado *et al*, *Biotechnol Bioeng.* 99: 475-484 (2008)), all are rapidly transported across the Rhesus monkey BBB in vivo. In the primate brain, the uptake of the fusion protein is approximately 1% of the injected dose (ID) (Boado *et al*, *Biotechnol Bioeng.* 99: 474-484 (2008)),. Since the weight of the human brain is about 10-fold greater than the weight of the Rhesus monkey brain, it is expected that the brain uptake of the HIRMAb-GDNF fusion protein by the human brain will be about 0.1% of the ID.

Given an injection dose of 25 mg, the expected brain concentration of the fusion protein is about 25 ng/gram human brain, which is equivalent to 5 ng/gram of GDNF, since the fusion protein is about 20% GDNF and 80% HIRMAb. The concentration of GDNF in the human brain is 0.2-1 ng/gram (Wiesenhofer *et al*, *Acta Neuropathol. (Berl)* 99:131-137 (2000)). Therefore, the dosing of 25 mg of the HIRMAb-GDNF fusion protein to a 60 kg human could result in pharmacologically significant increase in GDNF in the brain. In experimental Parkinson's disease, pharmacological effects are achieved with just a 3-fold increase in brain GDNF concentration (Eslamboli *et al*, *J. Neurosci.* 25:769-777 (2005)). Aberrant neuronal sprouting is induced when the brain GDNF concentration is increased >100-fold (Eslamboli *et al*, *J. Neurosci.* 25:769-777 (2005)).). However, such large increases in brain GDNF will not be produced by therapeutic dosing of the HIRMAb-GDNF fusion protein in humans.

[00242]   In conclusion, these studies described the genetic engineering, transient expression in COS cells, and validation of a HIRMAb-GDNF fusion protein, which represents a re-engineering of this neurotrophin to enable transport across the human BBB in vivo. The fusion protein can be administered by systemic injection to humans for treatment of multiple neurologic disorders, including stroke, neural repair, Parkinson's disease, or motor neuron disease.

Example 16

Expression of HIRMAb-GDNF fusion protein following stable transfection of CHO cells

[00243]   A tandem vector (TV) encoding the HIRMAb-GDNF fusion protein was engineered as shown in Figure 37, and is designated HIRMAb-GDNF TV. The TV contains on a single piece of DNA the fusion heavy chain (HC), the light chain (LC), and the gene for DHFR. DNA sequencing of the entire 6,300+ nucleotides (nt) of the HIRMAb-GDNF TV using custom oligodeoxynucleotides (ODNs) showed the sequence was comprised of 6,342 nt (SEQ ID NO. 47), which included the following domains:

- 731 nt cytomegalovirus (CMV) promoter
- 9 nt Kozak sequence (GCCGCCACC)
- 705 nt open reading frame (orf) encoding the HIRMAb LC
- 291 nt bovine growth hormone (BGH) polyA (pA) sequence
- 23 nt linker
- 714 nt CMV promoter
- 9 nt Kozak sequence
- 1,797 ORF encoding the fusion gene of the HIRMAb HC and GDNF
- 296 nt BGH pA
- 254 SV40 promoter
- 9 nt Kozak sequence
- 564 murine DHFR orf
- 940 hepatitis B virus (HBV) pA

[00244]   The HIRMAb-GDNF TV also included the expression cassette encoding neo, the neomycin resistance gene, to enable selection with G418 (Figure 37). It was necessary to include the HC fusion gene, the LC gene, and the DHFR gene on a single piece of DNA, or tandem vector (Figure 37) to allow for equally high expression of all 3 genes in the transfected CHO cell.

[00245]   The HIRMAb-GDNF TV sequence encoded for a 579 amino acid (AA) HC fusion protein (SEQ ID NO 48), which was comprised of a 19 AA IgG signal peptide, the 443 AA HIRMAb HC, a 2 AA linker, and the 134 AA human GDNF. The predicted molecular weight (MW) of the non-glycosylated HC was 63,860 Daltons (Da) and the predicted isolectric point (pI) of the fusion HC protein was 9.03. The HIRMAb-GDNF TV sequence encoded for a 234 AA LC protein (SEQ ID NO 49), which was comprised of a 20 AA IgG signal peptide, and the 214 AA HIRMAb LC. The predicted MW of the LC was 23,398 Da and the predicted pI of the LC protein was 5.45. The HIRMAb-GDNF TV sequence encoded for a DHFR protein (SEQ ID NO 50), that had an AA sequence 100% identical with the known AA sequence of murine DHFR. The domain structure of the HC is shown in Figure 36 and the domain structure of the LC is shown in Figure 38.

Electroporation of CHO cells.

[00246]   DG44 CHO cells were grown in serum-free medium (SFM), containing 1X HT supplement (hypoxanthine and thymidine). DG44 CHO cells (5 x 10^6 viable cells) were electroporated with 5 µg PvuI-linearized TV plasmid DNA. The cell-DNA suspension is then incubated for 10 min on ice. Cells are electroporated with pre-set protocol for CHO cells, i.e., square wave with pulse of 15 msec and 160 volts. After electroporation, cells are incubated for 10 min on ice. The cell suspension is transferred to 50 ml culture medium and plated at 125 µl per well in 40 x 96-well plates (10,000 cells per well), and 4,000 wells per study.

Selection and amplification with methotrexate and screening IgG ELISA.

[00247]   Following electroporation (EP), the CHO cells were placed in the incubator at 37 °C and 8% CO_2. Owing to the presence of the *neo* gen in the TV, transfected cell lines are initially selected with G418. The TV also contains the gene for DHFR, so the transfected cells were also selected with 20 nM methotrexate (MTX) and HT-deficient medium. Once visible colonies are detected at about 21 days after EP, the conditioned medium was sampled for human IgG by ELISA. Plates were removed from the incubator and transferred to the sterile hood where 100 µL samples were taken from each well using a Precision Pipettor system and transferred into a sterile 96-well tissue culture plate, which was then used for the human IgG ELISA. The media taken from the EP plates for the ELISA was replaced with 100 µL of SFM and cells were returned to the incubator at 37 °C and 8% CO_2. Wells with high human IgG signals in the ELISA were transferred from the 96-well plate to a 24-well plate with 1 mL of SFM. The 24-well plates were returned to the incubator at 37 °C and 8% CO_2. The following week IgG ELISA was performed on the clones in the 24-well plates. This was repeated through the 6-well plates to T75 flasks and finally to 60 mL and 125 mL square plastic bottles on an orbital shaker. After the cells adapted to the 60 mL bottle on the orbital shaker at 120RPM they were transferred into a 125 mL plastic square bottle with 12 mL of SFM.

At this stage, the final MTX concentration was 80 nM, and the medium IgG concentration, which is a measure of HIRMAb-GDNF fusion protein in the medium was >10 mg/L at a cell density of 10^6/mL.

Dilutional cloning of CHO cells.

[00248]   Clones selected for dilutional cloning (DC) were removed from the orbital shaker in the incubator and transferred to the sterile hood. The cells were diluted to 500 mL in F-12K medium with 5% dialyzed fetal bovine serum (d-FBS) and penicillin/streptomycin, and the final dilution was 8 cells per mL, so that 4,000 wells in 40 x 96-well plates were plated at a cell density of 1 cell per well (CPW). After the cell suspension was prepared, within the sterile hood, a 125μL aliquot was dispensed into each well of a 96-well plate using an 8-channel pipettor or a Precision Pipettor system. The plates were then returned to the incubator at 37 °C and 8% CO_2. The cells diluted to 1 cell/well cannot survive without serum. On day 6 or 7, DC plates were removed from the incubator and transferred to the sterile hood where 125 μL of F-12K medium with 5% dialyzed fetal bovine serum (d-FBS) was added to each well. After this step, the selection media contained 5% d-FBS, 30 nM MTX and 0.25 mg/mL Geneticin. On day 21 after the initial 1 CPW plating, aliquots from each of the 4,000 wells were removed for human IgG ELISA, using robotics equipment. DC plates were removed from the incubator and transferred to the sterile hood, where 100 μL of media was removed per well of the 96-well plate and transferred into a new, sterile sample 96-well plate using an 8-channel pipettor or a Precision Pipettor system.

ELISA screening of 4,000 wells.

[00249]   On day 20 after the initial 1 CPW plating, 44 96-well Immunoassay plates were plated with 100 μL of 1 μg/mL solution of Primary antibody, a mouse anti-human IgG in 0.1M NaHCO3. Plates were incubated overnight in the 4 °C Revco refrigerator. The following day, the plates were washed with 1x TBST 5 times, and 100 μL of 1 μg/mL solution of secondary antibody and blocking buffer were added. Immulon plates were washed with 1x TBST 5 times. 100 μL of 1mg/mL of 4-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt in 0.1M glycine buffer were added to the Immulon 96-well Immunoassay plates using the BioTek uFill. Plates were read on a microplate reader. The assay produced IgG output data for 4,000 wells/experiment. The highest producing 24-48 wells were selected for further propagation.

Adaptation of cloned cells to serum-free medium (SFM).

[00250]   The highest producing 24-well plates from the 1 CPW DC transferred to the sterile hood were gradually subcloned through 6-well dishes, T75 flasks, and 125 mL square plastic bottles on an orbital shaker. During this process the serum was reduced to zero, at the final stage of centrifugation of the cells and resuspension in SFM.

Second round dilutional cloning.

[00251]   The above procedures were repeated with a second round of dilutional cloning, again at 0.5 cells/well (CPW). At this stage, approximately 40% of the wells showed any cell growth, and all wells showing growth also secreted human IgG. These results confirmed that on average only 1

cell is plated per well with these procedures, and that the CHO cell line originated from a single cell.

Downstream processing and 3-column purification of the HIRMAb-GDNF fusion protein.

[00252]   Following the second round of dilutional cloning, the highest producing cell line secreting the HIRMAb-GDNF fusion protein was propagated in serum-free medium to a total volume of 2,000 mL in several 1 L square plastic bottles on an orbital shaker. The HIRMAb-GDNF fusion protein was purified from the CHO cell conditioned medium using the following down-stream processing:

- Depth filtration with a 0.2 m^2 0.65 um GF filter in series with an 0.05 m^2 0.2 μm ultrafilter

- Volume reduction to 400 mL using a Pellicon-2 tangential flow filtration (TFF) system

- Ultra-filtration with a 0.2 mm ultra-filter and application to a 7 mL column of protein A . Following application to the column, the column was eluted with 1 M NaCl, which elutes DNA non-specifically absorbed to the column, and the product is eluted as a single peak with 0.1 M sodium acetate/pH=3.7. The acid eluate was neutralized with 1 M Tris base and concentrated to 5 mL.

- Cation exchange (CATEX) chromatography in bind-elute mode was performed with a 5 mL column of SP Sepharose FF equilibrated with 0.02 M MES and 0.05 M NaCl. The conductivity of the sample was reduced to <5 mS/cm prior to application to the column. The column was successively eluted with step gradients of 0.02 M MES/pH=5.5 containing 0.25 M NaCl, 0.35 M NaCl, 0.5 M NaCl, and 1M NaCl. The HIRMAb-GDNF fusion protein eluted in 0.5 M NaCl.

- Anion exchange (ANEX) chromatography in flow-through mode was performed with a 5 mL column of Q Sepharose FF equilibrated with 0.025 M MES/pH=5.5 and 0.05 M NaCl. The conductivity of the sample was reduced to <7 mS/cm. The HIRMAb-GDNF fusion protein eluted in the flow-through.

Biochemical characterization of 3-column purified CHO cell derived HIRMAb-GDNF fusion protein.

[00253]   The purity and potency of the CHO derived HIRMAb-GDNF fusion protein was assessed with the following procedures:

(a) SDS-PAGE. The HIRMAb-GDNF fusion protein was purified to homogeneity based on reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A characteristic "fingerprint" of the HIRMAb-GDNF fusion protein is the detection of 3 heavy chain glycoforms, which arise from differential glycosylation of the GDNF part of the fusion protein. This pattern is reproducible from run to run.

(b) GDNF and human IgG Western blot. The CHO derived HIRMAb-GDNF fusion protein was electrophoresed on a 12% SDS-PAGE gel and blotted to nitrocellulose for Western blotting with primary antibodies to either human IgG (left panel, Figure 39), or to human GDNF (right panel, Figure 39). Both the anti-human IgG antibody and the anti-human GDNF antibody reacted specifically with the heavy chain of the HIRMAb-GDNF fusion protein.

CH2 region and 2 sites within the GDNF region of the fusion protein heavy chain. The neutral monosaccharide content (moles monosaccharide/mole fusion protein) was 7.7 for N-acetyl glucosamine, 5.3 for mannose, 2.3 for galactose, 1.6 for fucose, and 0.19 for N-acetyl galactosamine. These 4 sugars are organized as 1 of 4 different terminal structures, designated G0, G1(1,6), G1(1,3), or G2, depending on whether there is 0, 1, or 2 terminal galactose moieties, respectively. The profile for AGT-190 is G0>G1>G2, which is typical of CHO-derived recombinant proteins, and indicative of relative reduced terminal galactosylation. The glycosylation of HIRMAb-GDNF fusion protein on at least 3 different asparagine residues explains the heterogeneity of the heavy chain on Western blotting (Figure 39).

(h) **Binding to human blood-brain barrier.** Capillaries, which form the BBB in vivo, were isolated from human autopsy brain in a purified preparation as shown by light microscopy (Figure 43). The HIRMAb-GDNF fusion protein was radio-labeled by tritiation, and a radio-receptor assay was performed to demonstrate binding of the labeled HIRMAb-GDNF fusion protein to the HIR at the human BBB, using the isolated human brain capillaries. There was a high level of binding of the HIRMAb-GDNF fusion protein to the human brain capillary and the binding was displaced by unlabeled murine HIRMAb (Figure 43B).

Example 17

Treatment of Parkinson's disease with the HIRMAb-GDNF fusion protein

[00254]   Parkinson's disease (PD) is a neurodegenerative condition that affects the dopaminergic neurons of the nigral-striatal tract. GDNF is a potent trophic factor for these dopaminergic neurons. The intra-cerebral injection of the GDNF protein into the brain of rats with experimental PD can protect these neurons, and blocks further axotomy of the fibers projecting from the substantia nigra to the striatum. Accordingly, recombinant human GDNF has been developed as a new therapeutic for PD. However, GDNF does not cross the BBB. Therefore, GDNF was administered via a trans-cranial route using intra-cerebroventricular (ICV) injection. However, GDNF delivered by ICV injection was found to not penetrate into the brain parenchyma and was ineffective in patients with PD, and this delivery approach was abandoned. Subsequently, GDNF has been administered by convection enhanced diffusion (CED). In this approach, a reservoir holding the GDNF is implanted in the abdomen, and a catheter is run through the skin from the reservoir to the brain via a trans-cranial passage. The reservoir has a pump that continuously pumps the GDNF fluid into the brain. However, this highly invasive procedure was found to lead to toxic effects in the brain, was not effective owing to lack of penetration of the GDNF to the nigra-striatal tract, and was abandoned. These failed trans-cranial approaches to the treatment of PD with GDNF highlighted the importance of re-engineering this neurotrophin so that the molecule can cross the BBB. The trans-BBB delivery route has two unique advantages. First, the GDNF can be given by a non-invasive systemic injection, such as an intravenous, subcutaneous, or intra-muscular injection, and no neurosurgical procedure is required. Second, the GDNF enters the brain via the trans-vascular route. Since every

neuron in the brain is perfused by its own blood vessel, the GDNF is delivered to every cell comprising the nigra-striatal tract of brain. The GDNF could be given on a weekly or bi-weekly basis to patients with PD at doses ranging from 1, 3, 10, 30, or 100 mg. The considerations in Example 7 suggest that 25-30 mg may be a preferred dose.

Example 18

Acute and chronic treatment of stroke with the HIRMAb-GDNF fusion protein

[00255]   GDNF is highly neuroprotective in experimental stroke (Kitagawa *et al, Stroke* 29:1417-1422 (1998)). Activation of the GDNF receptor, GFRα1, inhibits apoptosis in the neuron that is induced by an ischemic event. The demonstration of GDNF's neuroprotective effects in experimental stroke have uniformly involved intra-cerebral injection of the neurotrophin, because the GDNF does not cross the BBB, and because the BBB is intact in the first 5 hours after stroke, when the rescue of dying neurons is still possible. The HIRMAb-GDNF is a re-engineered form of GDNF that is both equi-potent with GDNF as a neuroprotective agent, and is able to cross the human BBB following systemic administration. Thus, the patient suffering from an acute stroke could be rapidly treated with intravenous HIRMAb-GDNF in the emergency room. The HIRMAb-GDNF fusion protein will rapidly enter the brain and activate GFRα1 receptors on neurons to inhibit the apoptosis cycle induced by the stroke. In addition, chronic treatment with systemic HIRMAb-GDNF fusion protein could be therapeutic in the rehabilitation phase of stroke, since the intra-cerebral infusion of GDNF into the post-stroke brain induces neurogenesis, and neural repair in the post-stroke period (Kobayashi et al, 2006).

Example 19

Treatment of motor neuron disease with the HIRMAb-GDNF fusion protein

[00256]   Motor neuron diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) cause progressive paralysis leading to premature death. GDNF is highly neuroprotective of spinal cord motor neurons (Bohn *supra*). However, it is unlikely that GDNF will be developed as a drug for ALS or SMA, given the failed clinical trials of other neurotrophic factors for these conditions. Both BDNF and ciliary neurotrophic factor (CNTF) were administered to ALS patients in the 1990s by subcutaneous administration. The subcutaneous administration phase III clinical trials failed, because BDNF and CNTF, like GDNF, do not cross the BBB. However, with the re-engineering of GDNF as the HIRMAb-GDNF fusion protein demonstrated herein, conditions such as ALS or SMA could now be amenable to treatment by systemic administration. The HIRMAb fusion proteins cross the blood-spinal cord barrier, which as been demonstrated by brain scanning of adult Rhesus monkeys following intravenous administration of the HIRMAb fusion protein (Boado *et al, Biotechnol. Bioeng,* 97(6):1376-1386 (2007); Boado *et al, Bioconjug. Chem.* 18:447-455 (2007)).

Example 20

Treatment of brain or spinal cord injury with the HIRMAb-GDNF fusion protein

[00257]   GDNF promotes neural repair in the period following acute experimental brain injury,
such as with a lateral fluid percussion injury (Bakshi *et al*, *Eur. J. Neurosci.* 23:2119-2134 (2006)).
GDNF does not cross the BBB, and the BBB is intact in the days/weeks after head injury, when the
brain attempts to heal from the acute insult. Therefore, the beneficial effects of GDNF on acute
head injury could only be demonstrated by the intra-cerebral injection of genetically modified stem
cells that secrete GDNF. Although the trans-cranial injection approach may be feasible in the rat
brain, the human brain is 1000-fold larger than a rat brain. Diffusion of the GDNF from the depot
injection site becomes limiting, and the GDNF cannot penetrate to the wound area, beyond the local
injection site. Alternatively, if the GDNF is delivered to brain via the trans-vascular route, then
every injured neuron in the brain is exposed to the GDNF. The latter is feasible with the weekly, bi-
weekly, or daily systemic administration of the HIRMAb-GDNF fusion protein during the post-
injury repair and rehabilitation period.

[00258]   Similarly, motor neurons of the spinal cord are responsive to GDNF, and GDNF can
promote neural repair following experimental spinal cord injury (Lu *et al*, *J. Neurotrauma* 19:1081-
1090 (2002)). However, because the opening of the BBB following spinal cord injury is only
transient, it is not possible to treat spinal cord injury with systemic GDNF administration. Instead,
the GDNF expressing plasmid DNA was injected directly into the rat spinal cord following the
acute spinal cord injury. However, the size of the spinal cord in humans is 1000-fold larger than
that of the rat. Therefore, it is not possible to distribute GDNF to the entire lesioned area following
direct spinal injection of the GDNF therapeutic in humans. Alternatively, GDNF is delivered to all
injured neurons in the spinal cord following a trans-vascular delivery, and this is possible with the
systemic administration of the HIRMAb-GDNF fusion protein.

Example 21

Treatment of drug and alchohol addiction with the HIRMAb-GDNF fusion protein

[00259]   The intra-cerebral injection of GDNF results in treatment of animals addicted to either
opioid, or non-opioid drugs. With respect to non-opioid drugs, the intra-cerebral injection of GDNF
decreases cocaine self-aministration in rats (Green-Sadan et al, Eur. J. Neurosci., 18: 2093-2098,
2003). Similarly, the intra-cerebral injection of GDNF decreases ethanol self-administration (He et
al, J. Neurosci., 25: 619-628, 2005). The GDNF has to be administered via a trans-cranial route,
because GDNF does not cross the BBB. The drug, ibogaine, decreases withdrawl symptoms to
chronic opioid drugs, cocaine, or alchohol, and works by increasing GDNF in regions of the brain
such as the ventral tegmental area (VTA) (He and Ron, FASEB J, 20: E1820-D1827, 2006).
Although ibogaine is a small molecule that crosses the BBB, its application in the treatment of
addiction is limited by severe side effects. GDNF is implicated in another study related to cocaine
addiction. Cocaine increases tyrosine hydroxylase in the VTA, and this effect of cocaine is blocked

by the intra-cerebral injection of GDNF (Messer et al, Neuron, 26: 247-257, 2000). With respect to methamphetamine addiction, a decrease in brain concentration of GDNF, such as occurs in GDNF knock-out heterozygote mice, results in increased methamphetamine self-administration (Yan et al, FASEB J, 21: 1994-2004, 2007). These forms of addiction could be treated by systemic administration of GDNF if this neurotrophin was re-formulated to cross the BBB, which is the case for the HIRMAb-GDNF fusion protein.

[00260]   While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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ggatagacac ttcttgtgta tgtacattga ccattaaaag gtgatcgcga ctcgagatg


<210> 8
<211> 119
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 8
catctcgagt cgcgatcacc ttttaatggt caatgtacat acacaagaag tgtctatcct
tatgaatcgc cagccaattc tcttttgct atccatggta agggcccgca cgtacgact


<210> 9
<211> 29
<212> DNA
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 9
atctcgcgag tatgcactct gaccctgcc


<210> 10
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 10
atctcgcgat caccttttaa tggtcaa


<210> 11
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 11
atggctagcg atatcggtac cgtatacgga tccctcgaga tg


<210> 12
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 12
catctcgagg gatccgtata cggtaccgat atcgctagcc at


<210> 13
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide
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<400> 13
gtgacaaaca cagacatagg atatc

<210> 14
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 14
atgctcgagc taacactctc ccct

<210> 15
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 15
atgaatattc caccatggaa tgcagc

<210> 16
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 16
ataggatcct caccttttaa tggtcaa

<210> 17
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 17
aaaaggccag gaaccgaatt cagatctcgt tgctggcgtt tt

<210> 18

```
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 18
aaaacgccag caacgagatc tgaattcggt tcctggcctt tt


<210> 19
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 19
atcgaattca agcttgcggc cgcgtataca gatctatc


<210> 20
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Oligonucleotide

<400> 20
gatagatctg tatacgcggc cgcaagcttg aattcgat


<210> 21
<211> 378
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<220>
<221> CDS
<222> (1)..(375)

<400> 21
ctg tct ccg ggt aaa tcg agt atg cac tct gac cct gcc cgt cga ggt
Leu Ser Pro Gly Lys Ser Ser Met His Ser Asp Pro Ala Arg Arg Gly
gag ctg agc gtg tgt gac agt att agt gag tgg gta acg gcg gca gac
Glu Leu Ser Val Cys Asp Ser Ile Ser Glu Trp Val Thr Ala Ala Asp
aaa aag act gca gtg gac atg tcg ggc ggg acg gtc aca gtc ctt gaa
Lys Lys Thr Ala Val Asp Met Ser Gly Gly Thr Val Thr Val Leu Glu
aag atc cct gta tca aaa ggc caa ctg aag caa tac ttc tac gag acc
```

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Lys Val Pro Val Ser Lys Gly Gln Leu Lys Gln Tyr Phe Tyr Glu Thr
aag tgc aat ccc atg ggt tac aca aaa gaa ggc tgc agg ggc ata gac
Lys Cys Asn Pro Met Gly Tyr Thr Lys Glu Gly Cys Arg Gly Ile Asp
aaa agg cat tgg aac tcc cag tgc cga act acc cag tcg tac gtg cgg
Lys Arg His Trp Asn Ser Gln Cys Arg Thr Thr Gln Ser Tyr Val Arg
gcc ctt acc atg gat agc aaa aag aga att ggc tgg cga ttc ata agg
Ala Leu Thr Met Asp Ser Lys Lys Arg Ile Gly Trp Arg Phe Ile Arg
ata gac act tct tgt gta tgt aca ttg acc att aaa agg tga
Ile Asp Thr Ser Cys Val Cys Thr Leu Thr Ile Lys Arg
```

```
<210> 22
<211> 125
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 22
Leu Ser Pro Gly Lys Ser Ser Met His Ser Asp Pro Ala Arg Arg Gly
Glu Leu Ser Val Cys Asp Ser Ile Ser Glu Trp Val Thr Ala Ala Asp
Lys Lys Thr Ala Val Asp Met Ser Gly Gly Thr Val Thr Val Leu Glu
Lys Val Pro Val Ser Lys Gly Gln Leu Lys Gln Tyr Phe Tyr Glu Thr
Lys Cys Asn Pro Met Gly Tyr Thr Lys Glu Gly Cys Arg Gly Ile Asp
Lys Arg His Trp Asn Ser Gln Cys Arg Thr Thr Gln Ser Tyr Val Arg
Ala Leu Thr Met Asp Ser Lys Lys Arg Ile Gly Trp Arg Phe Ile Arg
Ile Asp Thr Ser Cys Val Cys Thr Leu Thr Ile Lys Arg
```

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<210> 23
<211> 2711
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 23
tagtctttct cttcagtgac aaacacagac ataggatatt ccaccatgga atgcagctgg
gtcatgctct tcctcctgtc aggaactgca ggtgtccatt gccaggttca gctgcagcag
tctggacctg agctggtgaa gcctggggct ttagtgaaga tatcctgcaa ggcttctggt
tacaccttca caaactacga tatacactgg gtgaagcaga ggcctggaca gggacttgag
tggattggat ggatttatcc tggagatggt agtactaagt acaatgagaa attcaagggc
aaggccacac tgactgcaga caaatcctcc agcacagcct acatgcacct cagcagcctg
acttctgaga aatctgcagt ctatttctgt gcaagagagt gggcttactg gggccaaggg
actctggtca ctgtctctgc agctagcacc aagggcccat cggtcttccc cctggcaccc
tcctccaaga gcacctctgg gggcacagcg gccctgggct gcctggtcaa ggactacttc
cccgaaccgg tgacggtgtc gtggaactca ggcgccctga ccagcggcgt gcacaccttc
ccggctgtcc tacagtcctc aggactctac tccctcagca gcgtggtgac cgtgccctcc
agcagcttgg gcacccagac ctacatctgc aacgtgaatc acaagcccag caacaccaag
gtggacaaga aagttggtga gaggccagca cagggaggga gggtgtctgc tggaagccag
gctcagcgct cctgcctgga cgcatccgg ctatgcagcc ccagtccagg cagcaaggc
aggccccgtc tgcctcttca cccggaggcc tctgcccgcc ccactcatgc tcagggagag
ggtcttctgg cttttccccc aggctctggg caggcacagg ctaggtgccc ctaacccagg
ccctgcacac aaaggggcag gtgctgggct cagacctgcc aagagccata tccgggagga
ccctgcccct gacctaagcc caccccaaag gccaaactct ccactccctc agctcggaca
```

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ccttctctcc tcccagattc cagtaactcc caatcttctc tctgcagagc ccaaatcttg
tgacaaaact cacacatgcc caccgtgccc aggtaagcca gcccaggcct cgccctccag
ctcaaggcgg gacaggtgcc ctagagtagc ctgcatccag ggacaggccc cagccgggtg
ctgacacgtc cacctccatc tcttcctcag cacctgaact cctgggggga ccgtcagtct
tcctcttccc cccaaaaccc aaggacaccc tcatgatctc ccggacccct gaggtcacat
gcgtggtggt ggacgtgagc cacgaagacc ctgaggtcaa gttcaactgg tacgtggacg
gcgtggaggt gcataatgcc aagacaaagc cgcgggagga gcagtacaac agcacgtacc
gtgtggtcag ggtcctcacc gtcctgcacc aggactggct gaatggcaag gagtacaagt
gcaaggtctc caacaaagcc ctcccagccc ccatcgagaa aaccatctcc aaagccaaag
gtgggacccg tggggtgcga gggccacatg gacagaggcc ggctcggccc accctctgcc
ctgagagtga ccgctgtacc aacctctgtc cctacagggc agccccgaga accacaggtg
tacaccctgc ccccatcccg ggatgagctg accaagaacc aggtcagcct gacctgcctg
gtcaaaggct tctatcccag cgacatcgcc gtggagtggg agagcaatgg gcagccggag
aacaactaca agaccacgcc tcccgtgctg gactccgacg gctccttctt cctctacagc
aagctcaccg tggacaagag caggtggcag caggggaacg tcttctcatg ctccgtgatg
catgaggctc tgcacaacca ctacacgcag aagagcctct ccctgtctcc gggtaaatcg
agtatgcact ctgaccctgc ccgtcgaggt gagctgagcg tgtgtgacag tattagtgag
tgggtaacgg cggcagacaa aaagactgca gtggacatgt cgggcgggac ggtcacagtc
cttgaaaagg tccctgtatc aaaaggccaa ctgaagcaat acttctacga gaccaagtgc
aatcccatgg gttacacaaa agaaggctgc aggggcatag acaaaaggca ttggaactcc
cagtgccgaa ctacccagtc gtacgtgcgg gcccttacca tggatagcaa aaagagaatt
ggctggcgat tcataaggat agacacttct tgtgtatgta cattgaccat taaaaggtga
tcgattttgc gacggccggc aagcccccgc tccccgggct ctcgcggtcg cacgaggatg
cttggcacgt accccctgta catacttccc gggcgcccag catggaaata aagcacccag
cgctgccctg ggccctgcg agactgtgat ggttctttcc acgggtcagg ccgagtctga
ggcctgagtg gcatgaggga ggcagagcgg gtcccactgt ccccacactg gcccaggctg
tgcaggtgtg cctgggccgc ctagggtggg gctcagccag gggctgccct cggcagggtg
ggggatttgc c
```

```
<210> 24
<211> 582
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 24
Met Glu Cys Ser Trp Val Met Leu Phe Leu Leu Ser Gly Thr Ala Gly
Val His Cys Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
Pro Gly Ala Leu Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
Thr Asn Tyr Asp Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
Glu Trp Ile Gly Trp Ile Tyr Pro Gly Asp Gly Ser Thr Lys Tyr Asn
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Lys Ser Ala Val
Tyr Phe Cys Ala Arg Glu Trp Ala Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
```

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Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Arg Val
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Ser
Met His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser
Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met
Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr
Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln
Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys
Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys
Thr Leu Thr Ile Lys Arg
```


<210> 25
<211> 582
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 25
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Met Glu Cys Ser Trp Val Met Leu Phe Leu Leu Ser Gly Thr Ala Gly
Val His Cys Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
Pro Gly Ala Leu Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
Thr Asn Tyr Asp Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
Glu Trp Ile Gly Trp Ile Tyr Pro Gly Asp Gly Ser Thr Lys Tyr Asn
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Lys Ser Ala Val
Tyr Phe Cys Ala Arg Glu Trp Ala Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Ser
```

```
Met His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser
Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met
Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr
Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln
Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys
Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys
Thr Leu Thr Ile Lys Arg
```

<210> 26
<211> 1757
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 26
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attccaccat ggaatgcagc tgggtcatgc tcttcctcct gtcaggaact gcaggtgtcc
attgccaggt tcagctgcag cagtctggac ctgagctggt gaagcctggg gctttagtga
agatatcctg caaggcttct ggttacacct tcacaaacta cgatatacac tgggtgaagc
agaggcctgg acagggactt gagtggattg gatggattta tcctggagat ggtagtacta
agtacaatga gaaattcaag ggcaaggcca cactgactgc agacaaatcc tccagcacag
cctacatgca cctcagcagc ctgacttctg agaaatctgc agtctatttc tgtgcaagag
agtgggctta ctggggccaa gggactctgg tcactgtctc tgcagctagc accaagggcc
catcggtctt ccccctggca ccctcctcca gagcacctc tggggcaca gcggccctgg
gctgcctggt caaggactac ttccccgaac cggtgacggt gtcgtggaac tcaggcgccc
tgaccagcgg cgtgcacacc ttcccggctg tcctacagtc ctcaggactc tactccctca
gcagcgtggt gaccgtgccc tccagcagct tgggcaccca gacctacatc tgcaacgtga
atcacaagcc cagcaacacc aaggtggaca agaaagttga gcccaaatct tgtgacaaaa
ctcacacatg cccaccgtgc ccagcacctg aactcctggg gggaccgtca gtcttcctct
tcccccaaa acccaaggac accctcatga tctcccggac ccctgaggtc acatgcgtgg
tggtggacgt gagccacgaa gaccctgagg tcaagttcaa ctggtacgtg gacggcgtgg
aggtgcataa tgccaagaca aagccgcggg aggagcagta caacagcacg taccgtgtgg
tcagcgtcct caccgtcctg caccaggact ggctgaatgg caaggagtac aagtgcaagg
tctccaacaa agccctccca gcccccatcg agaaaaccat ctccaaagcc aaagggcagc
cccgagaacc acaggtgtac accctgcccc catcccggga tgagctgacc aagaaccagg
tcagcctgac ctgcctggtc aaaggcttct atcccagcga catcgccgtg gagtgggaga
gcaatgggca gccggagaac aactacaaga ccacgcctcc cgtgctggac tccgacggct
ccttcttcct ctacagcaag ctcaccgtgg acaagagcag gtggcagcag gggaacgtct
tctcatgctc cgtgatgcat gaggctctgc acaaccacta cacgcagaag agcctctccc
tgtctccggg taaatcgagt atgcactctg accctgcccg tcgaggtgag ctgagcgtgt
gtgacagtat tagtgagtgg gtaacggcgg cagacaaaaa gactgcagtg gacatgtcgg
gcgggacggt cacagtcctt gaaaaggtcc ctgtatcaaa aggccaactg aagcaatact
tctacgagac caagtgcaat cccatgggtt acacaaaaga aggctgcagg ggcatagaca
aaaggcattg gaactcccag tgccgaacta cccagtcgta cgtgcgggcc cttaccatgg
atagcaaaaa gagaattggc tggcgattca taaggataga cacttcttgt gtatgtacat
tgaccattaa aaggtga
```

<210> 27
<211> 1749

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<220>
<221> CDS
<222> (1)..(1746)

<400> 27
atg gaa tgc agc tgg gtc atg ctc ttc ctc ctg tca gga act gca ggt
Met Glu Cys Ser Trp Val Met Leu Phe Leu Leu Ser Gly Thr Ala Gly
gtc cat tgc cag gtt cag ctg cag cag tct gga cct gag ctg gtg aag
Val His Cys Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
cct ggg gct tta gtg aag ata tcc tgc aag gct tct ggt tac acc ttc
Pro Gly Ala Leu Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
aca aac tac gat ata cac tgg gtg aag cag agg cct gga cag gga ctt
Thr Asn Tyr Asp Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
gag tgg att gga tgg att tat cct gga gat ggt agt act aag tac aat
Glu Trp Ile Gly Trp Ile Tyr Pro Gly Asp Gly Ser Thr Lys Tyr Asn
gag aaa ttc aag ggc aag gcc aca ctg act gca gac aaa tcc tcc agc
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
aca gcc tac atg cac ctc agc agc ctg act tct gag aaa tct gca gtc
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Lys Ser Ala Val
tat ttc tgt gca aga gag tgg gct tac tgg ggc caa ggg act ctg gtc
Tyr Phe Cys Ala Arg Glu Trp Ala Tyr Trp Gly Gln Gly Thr Leu Val
act gtc tct gca gct agc acc aag ggc cca tcg gtc ttc ccc ctg gca
Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
ccc tcc tcc aag agc acc tct ggg ggc aca gcg gcc ctg ggc tgc ctg
Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
gtc aag gac tac ttc ccc gaa ccg gtg acg gtg tcg tgg aac tca ggc
Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
gcc ctg acc agc ggc gtg cac acc ttc ccg gct gtc cta cag tcc tca
Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
gga ctc tac tcc ctc agc agc gtg gtg acc gtg ccc tcc agc agc ttg
Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
ggc acc cag acc tac atc tgc aac gtg aat cac aag ccc agc aac acc
Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
aag gtg gac aag aaa gtt gag ccc aaa tct tgt gac aaa act cac aca
Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
gag gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
ctc acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
aag gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
aaa gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
```

```
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tcg agt
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Ser
atg cac tct gac cct gcc cgt cga ggt gag ctg agc gtg tgt gac agt
Met His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser
att agt gag tgg gta acg gcg gca gac aaa aag act gca gtg gac atg
Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met
tcg ggc ggg acg gtc aca gtc ctt gaa aag gtc cct gta tca aaa ggc
Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
caa ctg aag caa tac ttc tac gag acc aag tgc aat ccc atg ggt tac
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr
aca aaa gaa ggc tgc agg ggc ata gac aaa agg cat tgg aac tcc cag
Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln
tgc cga act acc cag tcg tac gtg cgg gcc ctt acc atg gat agc aaa
Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys
aag aga att ggc tgg cga ttc ata agg ata gac act tct tgt gta tgt
Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys
aca ttg acc att aaa agg tga
Thr Leu Thr Ile Lys Arg
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<210> 28
<211> 582
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 28
Met Glu Cys Ser Trp Val Met Leu Phe Leu Leu Ser Gly Thr Ala Gly
Val His Cys Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
Pro Gly Ala Leu Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
Thr Asn Tyr Asp Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
Glu Trp Ile Gly Trp Ile Tyr Pro Gly Asp Gly Ser Thr Lys Tyr Asn
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Lys Ser Ala Val
Tyr Phe Cys Ala Arg Glu Trp Ala Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro

Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
```

```
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Ser
Met His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser
Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met
Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr
Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln
Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys
Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys
Thr Leu Thr Ile Lys Arg
```

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<210> 29
<211> 714
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 29
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agagtcagtc tcacttgtcg ggcaagtcag gacattggtg gtaacttata ctggcttcag
caggggaccag atggaactat taaacgcctg atctacgcca catccagttt agattctggt
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aacttctatc ccagagaggc caaagtacag tggaaggtgg ataacgccct ccaatcgggt
aactcccagg agagtgtcac agagcaggac agcaaggaca gcacctacag cctcagcagc
accctgacgc tgagcaaagc agactacgag aaacacaaag tctacgcctg cgaagtcacc
catcagggcc tgagctcgcc cgtcacaaag agcttcaaca ggggagagtg ttag
```

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<210> 30
<211> 705
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<220>
<221> CDS
<222> (1)..(702)

<400> 30
atg gag aca gac aca ctc ctg cta tgg ctc ttg ttg ctc atg ttt cca
Met Glu Thr Asp Thr Leu Leu Leu Trp Leu Leu Leu Leu Met Phe Pro
```

```
ggt acc aga tgt gac atc cag atg acc cag tct cca tcc tcc tta tct
Gly Thr Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
gcc tct ctg gga gaa aga gtc agt ctc act tgt cgg gca agt cag gac
Ala Ser Leu Gly Glu Arg Val Ser Leu Thr Cys Arg Ala Ser Gln Asp
att ggt ggt aac tta tac tgg ctt cag cag gga cca gat gga act att
Ile Gly Gly Asn Leu Tyr Trp Leu Gln Gln Gly Pro Asp Gly Thr Ile
aaa cgc ctg atc tac gcc aca tcc agt tta gat tct ggt gtc ccc aaa
Lys Arg Leu Ile Tyr Ala Thr Ser Ser Leu Asp Ser Gly Val Pro Lys
agg ttc agt ggc agt agg tct ggg tca gat tat tct ctc acc atc agc
Arg Phe Ser Gly Ser Arg Ser Gly Ser Asp Tyr Ser Leu Thr Ile Ser
agc ctt gag tct gaa gat ttt gta gac tat tac tgt cta cag tat tct
Ser Leu Glu Ser Glu Asp Phe Val Asp Tyr Tyr Cys Leu Gln Tyr Ser
agt tct ccg tgg acg ttc ggt gga gcg aca aag atg gaa ata aaa cga
Ser Ser Pro Trp Thr Phe Gly Gly Ala Thr Lys Met Glu Ile Lys Arg
act gtg gct gca cca tct gtc ttc atc ttc ccg cca tct gat gag cag
Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
ttg aaa tct gga act gcc tct gtt gtg tgc ctg ctg aat aac ttc tat
Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
ccc aga gag gcc aaa gta cag tgg aag gtg gat aac gcc ctc caa tcg
Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
ggt aac tcc cag gag agt gtc aca gag cag gac agc aag gac agc acc
Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
tac agc ctc agc agc acc ctg acg ctg agc aaa gca gac tac gag aaa
Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
cac aaa gtc tac gcc tgc gaa gtc acc cat cag ggc ctg agc tcg ccc
His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
gtc aca aag agc ttc aac agg gga gag tgt tag
Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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```
<210> 31
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 31
Met Glu Thr Asp Thr Leu Leu Leu Trp Leu Leu Leu Leu Met Phe Pro
Gly Thr Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
Ala Ser Leu Gly Glu Arg Val Ser Leu Thr Cys Arg Ala Ser Gln Asp
Ile Gly Gly Asn Leu Tyr Trp Leu Gln Gln Gly Pro Asp Gly Thr Ile
Lys Arg Leu Ile Tyr Ala Thr Ser Ser Leu Asp Ser Gly Val Pro Lys
Arg Phe Ser Gly Ser Arg Ser Gly Ser Asp Tyr Ser Leu Thr Ile Ser
Ser Leu Glu Ser Glu Asp Phe Val Asp Tyr Tyr Cys Leu Gln Tyr Ser
Ser Ser Pro Trp Thr Phe Gly Gly Ala Thr Lys Met Glu Ile Lys Arg
Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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<210> 32
<211> 6505
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

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aaatggcccg cctggctgac cgcccaacga ccccgccca ttgacgtcaa taatgacgta
tgttcccata gtaacgccaa tagggacttt ccattgacgt caatgggtgg agtatttacg
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gcagtacatc aatgggcgtg gatagcggtt tgactcacgg ggatttccaa gtctccaccc
cattgacgtc aatgggagtt tgttttggca ccaaaatcaa cgggactttc caaaatgtcg
taacaactcc gccccattga cgcaaatggg cggtaggcgt gtacggtggg aggtctatat
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aattagtcag ccatggggcg gagaatgggc ggaactgggc ggagttaggg gcgggatggg
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gcgtaagagc tcggtacctc cctac
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98

<400> 33
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Met Glu Cys Ser Trp Val Met Leu Phe Leu Leu Ser Gly Thr Ala Gly
gtc cat tgc cag gtt cag ctg cag cag tct gga cct gag ctg gtg aag
Val His Cys Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
cct ggg gct tta gtg aag ata tcc tgc aag gct tct ggt tac acc ttc
Pro Gly Ala Leu Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe

aca aac tac gat ata cac tgg gtg aag cag agg cct gga cag gga ctt
Thr Asn Tyr Asp Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
gag tgg att gga tgg att tat cct gga gat ggt agt act aag tac aat
Glu Trp Ile Gly Trp Ile Tyr Pro Gly Asp Gly Ser Thr Lys Tyr Asn
gag aaa ttc aag ggc aag gcc aca ctg act gca gac aaa tcc tcc agc
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
aca gcc tac atg cac ctc agc agc ctg act tct gag aaa tct gca gtc
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Lys Ser Ala Val
tat ttc tgt gca aga gag tgg gct tac tgg ggc caa ggg act ctg gtc
Tyr Phe Cys Ala Arg Glu Trp Ala Tyr Trp Gly Gln Gly Thr Leu Val
act gtc tct gca gct agc acc aag ggc cca tcg gtc ttc ccc ctg gca
Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
ccc tcc tcc aag agc acc tct ggg ggc aca gcg gcc ctg ggc tgc ctg
Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
gtc aag gac tac ttc ccc gaa ccg gtg acg gtg tcg tgg aac tca ggc
Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
gcc ctg acc agc ggc gtg cac acc ttc ccg gct gtc cta cag tcc tca
Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
gga ctc tac tcc ctc agc agc gtg gtg acc gtg ccc tcc agc agc ttg
Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
ggc acc cag acc tac atc tgc aac gtg aat cac aag ccc agc aac acc
Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
aag gtg gac aag aaa gtt gag ccc aaa tct tgt gac aaa act cac aca
Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
gag gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
ctc acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys

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Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
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Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
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Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
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Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
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Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
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Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
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Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Ser
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Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met
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Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
caa ctg aag caa tac ttc tac gag acc aag tgc aat ccc atg ggt tac
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr
aca aaa gaa ggc tgc agg ggc ata gac aaa agg cat tgg aac tcc cag
Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln
tgc cga act acc cag tcg tac gtg cgg gcc ctt acc atg gat agc aaa
Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys
aag aga att ggc tgg cga ttc ata agg ata gac act tct tgt gta tgt
Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys
aca ttg acc att aaa agg tga
Thr Leu Thr Ile Lys Arg
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<210> 34
<211> 582
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 34
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Val His Cys Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
Pro Gly Ala Leu Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
Thr Asn Tyr Asp Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
Glu Trp Ile Gly Trp Ile Tyr Pro Gly Asp Gly Ser Thr Lys Tyr Asn
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
Thr Ala Tyr Met His Leu Ser Ser Leu Thr Ser Glu Lys Ser Ala Val
Tyr Phe Cys Ala Arg Glu Trp Ala Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
```

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Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Ser
Met His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser
Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met
Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr
Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln
Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys
Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys
Thr Leu Thr Ile Lys Arg
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<210> 35
<211> 705
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<220>
<221> CDS
<222> (1)..(702)

<400> 35
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Met Glu Thr Asp Thr Leu Leu Leu Trp Leu Leu Leu Leu Met Phe Pro
ggt acc aga tgt gac atc cag atg acc cag tct cca tcc tcc tta tct
Gly Thr Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
gcc tct ctg gga gaa aga gtc agt ctc act tgt cgg gca agt cag gac
Ala Ser Leu Gly Glu Arg Val Ser Leu Thr Cys Arg Ala Ser Gln Asp
att ggt ggt aac tta tac tgg ctt cag cag gga cca gat gga act att
Ile Gly Gly Asn Leu Tyr Trp Leu Gln Gln Gly Pro Asp Gly Thr Ile
aaa cgc ctg atc tac gcc aca tcc agt tta gat tct ggt gtc ccc aaa
Lys Arg Leu Ile Tyr Ala Thr Ser Ser Leu Asp Ser Gly Val Pro Lys
agg ttc agt ggc agt agg tct ggg tca gat tat tct ctc acc atc agc
Arg Phe Ser Gly Ser Arg Ser Gly Ser Asp Tyr Ser Leu Thr Ile Ser
agc ctt gag tct gaa gat ttt gta gac tat tac tgt cta cag tat tct
Ser Leu Glu Ser Glu Asp Phe Val Asp Tyr Tyr Cys Leu Gln Tyr Ser
agt tct ccg tgg acg ttc ggt gga gcg aca aag atg gaa ata aaa cga
Ser Ser Pro Trp Thr Phe Gly Gly Ala Thr Lys Met Glu Ile Lys Arg
act gtg gct gca cca tct gtc ttc atc ttc ccg cca tct gat gag cag
Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
ttg aaa tct gga act gcc tct gtt gtg tgc ctg ctg aat aac ttc tat
Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
ccc aga gag gcc aaa gta cag tgg aag gtg gat aac gcc ctc caa tcg
```

```
Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
ggt aac tcc cag gag agt gtc aca gag cag gac agc aag gac agc acc
Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
tac agc ctc agc agc acc ctg acg ctg agc aaa gca gac tac gag aaa
Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
cac aaa gtc tac gcc tgc gaa gtc acc cat cag ggc ctg agc tcg ccc
His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
gtc aca aag agc ttc aac agg gga gag tgt tag
Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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<210> 36
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 36
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Gly Thr Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
Ala Ser Leu Gly Glu Arg Val Ser Leu Thr Cys Arg Ala Ser Gln Asp
Ile Gly Gly Asn Leu Tyr Trp Leu Gln Gln Gly Pro Asp Gly Thr Ile
Lys Arg Leu Ile Tyr Ala Thr Ser Ser Leu Asp Ser Gly Val Pro Lys
Arg Phe Ser Gly Ser Arg Ser Gly Ser Asp Tyr Ser Leu Thr Ile Ser
Ser Leu Glu Ser Glu Asp Phe Val Asp Tyr Tyr Cys Leu Gln Tyr Ser
Ser Ser Pro Trp Thr Phe Gly Gly Ala Thr Lys Met Glu Ile Lys Arg
Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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<210> 37
<211> 564
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(561)

<400> 37
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atg gtt cga cca ttg aac tgc atc gtc gcc gtg tcc caa aat atg ggg
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att ggc aag aac gga gac cga ccc tgg cct ccg ctc agg aac gag ttc
Ile Gly Lys Asn Gly Asp Arg Pro Trp Pro Pro Leu Arg Asn Glu Phe
aag tac ttc caa aga atg acc aca acc tct tca gtg gaa ggt aaa cag
Lys Tyr Phe Gln Arg Met Thr Thr Thr Ser Ser Val Glu Gly Lys Gln
aat ctg gtg att atg ggt agg aaa acc tgg ttc tcc att cct gag aag
Asn Leu Val Ile Met Gly Arg Lys Thr Trp Phe Ser Ile Pro Glu Lys
aat cga cct tta aag gac aga att aat ata gtt ctc agt aga gaa ctc
Asn Arg Pro Leu Lys Asp Arg Ile Asn Ile Val Leu Ser Arg Glu Leu
aaa gaa cca cca cga gga gct cat ttt ctt gcc aaa agt ttg gat gat
Lys Glu Pro Pro Arg Gly Ala His Phe Leu Ala Lys Ser Leu Asp Asp
```

```
Lys Glu Pro Pro Arg Gly Ala His Phe Leu Ala Lys Ser Leu Asp Asp
gcc tta aga ctt att gaa caa ccg gaa ttg gca agt aaa gta gac atg
Ala Leu Arg Leu Ile Glu Gln Pro Glu Leu Ala Ser Lys Val Asp Met
gtt tgg ata gtc gga ggc agt tct gtt tac cag gaa gcc atg aat caa
Val Trp Ile Val Gly Gly Ser Ser Val Tyr Gln Glu Ala Met Asn Gln
cca ggc cac ctc aga ctc ttt gtg aca agg atc atg cag gaa ttt gaa
Pro Gly His Leu Arg Leu Phe Val Thr Arg Ile Met Gln Glu Phe Glu
agt gac acg ttt ttc cca gaa att gat ttg ggg aaa tat aaa ctt ctc
Ser Asp Thr Phe Phe Pro Glu Ile Asp Leu Gly Lys Tyr Lys Leu Leu
cca gaa tac cca ggc gtc ctc tct gag gtc cag gag gaa aaa ggc atc
Pro Glu Tyr Pro Gly Val Leu Ser Glu Val Gln Glu Glu Lys Gly Ile
aag tat aag ttt gaa gtc tac gag aag aaa gac taa
Lys Tyr Lys Phe Glu Val Tyr Glu Lys Lys Asp
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<210> 38
<211> 187
<212> PRT
<213> Mus musculus

<400> 38
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Ile Gly Lys Asn Gly Asp Arg Pro Trp Pro Pro Leu Arg Asn Glu Phe
Lys Tyr Phe Gln Arg Met Thr Thr Thr Ser Ser Val Glu Gly Lys Gln
Asn Leu Val Ile Met Gly Arg Lys Thr Trp Phe Ser Ile Pro Glu Lys
Asn Arg Pro Leu Lys Asp Arg Ile Asn Ile Val Leu Ser Arg Glu Leu
Lys Glu Pro Pro Arg Gly Ala His Phe Leu Ala Lys Ser Leu Asp Asp
Ala Leu Arg Leu Ile Glu Gln Pro Glu Leu Ala Ser Lys Val Asp Met
Val Trp Ile Val Gly Gly Ser Ser Val Tyr Gln Glu Ala Met Asn Gln
Pro Gly His Leu Arg Leu Phe Val Thr Arg Ile Met Gln Glu Phe Glu
Ser Asp Thr Phe Phe Pro Glu Ile Asp Leu Gly Lys Tyr Lys Leu Leu
Pro Glu Tyr Pro Gly Val Leu Ser Glu Val Gln Glu Glu Lys Gly Ile
Lys Tyr Lys Phe Glu Val Tyr Glu Lys Lys Asp
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<210> 39
<211> 119
<212> PRT
<213> Homo sapiens

<400> 39
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His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser Ile
Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Met Ser
Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly Gln
Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr Thr
Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln Cys
Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys Lys
Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val Cys Thr
Leu Thr Ile Lys Arg Gly Arg
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<210> 40
<211> 25
<212> DNA
<213> artificial sequence

<220>
<223> Description of Artificial Sequence: Synthetic Oligonucleotide

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<400>    40
ATGAAGTTATGGGATGTCGTGGCTG


<210>        41
<211>        25
<212>        DNA
<213>        artificial sequence

<220>
<223>        Description of Artificial Sequence: Synthetic
Oligonucleotide

<400>        41
TCAGATACATCCACACCTTTTAGCG


<210>        42
<211>        26
<212>        DNA
<213>        artificial sequence

<220>
<223>        Description of Artificial Sequence: Synthetic
Oligonucleotide

<400>        42
CATCACCAGATAAACAAATGGCAGTG


<210>        43
<211>        636
<212>        DNA
<213>        artificial sequence

<220>
<223>        Description of Artificial Sequence: Synthetic DNA

<400>        43
ATGAAGTTATGGGATGTCGTGGCTGTCTGCCTGGTGCTGCTCCACACCGCGTCCGCCTTCCCGCTGCCCGCC
GGTAAGAGGCCTCCCGAGGCGCCCGCCGAAGACCGCTCCCTCGGCCGCCGCCGCGCGCCCTTCGCGCTGAGC
AGTGACTCAAATATGCCAGAGGATTATCCTGATCAGTTCGATGATGTCATGGATTTTATTCAAGCCACCATT
AAAAGACTGAAAAGGTCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCA
GCTGCCAACCCAGAGAATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGTTGTGTCTTA
ACTGCAATACATTTAAATGTCACTGACTTGGGTCTGGGCTATGAAACCAAGGAGGAACTGATTTTTAGGTAC
TGCAGCGGCTCTTGCGATGCAGCTGAGACAACGTACGACAAAATATTGAAAAACTTATCCAGAAATAGAAGG
CTGGTGAGTGACAAAGTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGATGATGACCTGTCGTTTTTAGAT
GATAACCTGGTTTACCATATTCTAAGAAAGCATTCCGCTAAAAGGTGTGGATGTATCTGA


<210>        44
<211>        211
<212>        PRT
<213>        artificial sequence

<220>
<223>        Description of Artificial Sequence: Synthetic Protein
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<400>   44
MKLWDVVAVCLVLLHTASAFPLPAGKRPPEAPAEDRSLGRRRAPFALSSDSNMPEDYPDQFDDVMDFIQATI
KRLKRSPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYETKEELIFRY
CSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

<210>   45
<211>   1797
<212>   DNA
<213>   artificial sequence

<220>
<223>   Description of Artificial Sequence: Synthetic DNA

<400>   45
ATGGACTGGACCTGGAGGGTGTTCTGCCTGCTTGCAGTGGCCCCCGGAGCCCACAGCCAGGTTCAG
CTGCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTTAGTGAAGATATCCTGCAAGGCTTCT
GGTTACACCTTCACAAACTACGATATACACTGGGTGAAGCAGAGGCCTGGACAGGGACTTGAGTGG
ATTGGATGGATTTATCCTGGAGATGGTAGTACTAAGTACAATGAGAAATTCAAGGGCAAGGCCACA
CTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCACCTCAGCAGCCTGACTTCTGAGAAATCT
GCAGTCTATTTCTGTGCAAGAGAGTGGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA
GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACA
GCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGC
GCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGC
AGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAG
CCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCA
CCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGAC
ACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCT
GAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG
GAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAAT
GGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCC
AAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC
AAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGG
GAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCC
TTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGC
TCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA
AGTTCATCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCAGCT
GCCAACCCAGAGAATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGTTGTGTC
TTAACTGCAATACATTTAAATGTCACTGACTTGGGTCTGGGCTATGAAACCAAGGAGGAACTGATT
TTTAGGTACTGCAGCGGCTCTTGCGATGCAGCTGAGACAACGTACGACAAAATATTGAAAAACTTA
TCCAGAAATAGAAGGCTGGTGAGTGACAAAGTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGAT
GATGACCTGTCGTTTTTAGATGATAACCTGGTTTACCATATTCTAAGAAAGCATTCCGCTAAAAGG
TGTGGATGTATCTGA

<210>   46
<211>   598
<212>   PRT
<213>   artificial sequence

<220>
<223>   Description of Artificial Sequence: Synthetic Protein

<400>   46

MDWTWRVFCLLAVAPGAHSQVQLQQSGPELVKPGALVKISCKASGYTFTNYDIHWVKQRPGQGLEWIGWIYP
GDGSTKYNEKFKGKATLTADKSSSTAYMHLSSLTSEKSAVYFCAREWAYWGQGTLVTVSAASTKGPSVFPLA
PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGKSSSPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRG
CVLTAIHLNVTDLGLGYETKEELIFRYCSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLS
FLDDNLVYHILRKHSAKRCGCI


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<210>      47
<211>      6342
<212>      DNA
<213>      artificial sequence

<220>
<223>      Description of Artificial Sequence: Synthetic DNA

<400>      47
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CCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATT
GACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT
GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTAC
ATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGG
TGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAG
TCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAA
TGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
TAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGAC
TCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCA
CTAGTCCAGTGTGGTGGAATTCTGCAGGCCGCCACCATGGAGACCCCCGCCCAGCTGCTGTTCC
TGTTGCTGCTTTGGCTTCCAGATACTACCGGCGACATCCAGATGACCCAGTCTCCATCCTCCTT
ATCTGCCTCTCTGGGAGAAAGAGTCAGTCTCACTTGTCGGGCAAGTCAGGACATTGGTGGTAAC
TTATACTGGCTTCAGCAGGGACCAGATGGAACTATTAAACGCCTGATCTACGCCACATCCAGTT
TAGATTCTGGTGTCCCCAAAAGGTTCAGTGGCAGTAGGTCTGGGTCAGATTATTCTCTCACCAT
CAGCAGCCTTGAGTCTGAAGATTTTGTAGACTATTACTGTCTACAGTATTCTAGTTCTCCGTGG
ACGTTCGGTGGAGGCACAAAGATGGAAATAAAACGAACTGTGGCTGCACCATCTGTCTTCATCT
TCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTT
CTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAG
GAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGA
GCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTC
GCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAGCTCGAGTCTAGAGGGCCCGTTTAAACC
CGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGC
CTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATC
GCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAG
GATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAA
GAACCAGCCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGT
AATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGT
AAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTT
CCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTG
CCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGG
TAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTAC
ATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTG
GATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTT
TTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATG
GGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCA
CTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTT
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AAACGGGCCCTCTAGACTCGAGCGGCCGCCACTGTGCTGGAGCCGCCACCATGGACTGGACCTG
GAGGGTGTTCTGCCTGCTTGCAGTGGCCCCCGGAGCCCACAGCCAGGTTCAGCTGCAGCAGTCT
GGACCTGAGCTGGTGAAGCCTGGGGCTTTAGTGAAGATATCCTGCAAGGCTTCTGGTTACACCT
TCACAAACTACGATATACACTGGGTGAAGCAGAGGCCTGGACAGGGACTTGAGTGGATTGGATG
GATTTATCCTGGAGATGGTAGTACTAAGTACAATGAGAAATTCAAGGGCAAGGCCACACTGACT
GCAGACAAATCCTCCAGCACAGCCTACATGCACCTCAGCAGCCTGACTTCTGAGAAATCTGCAG
TCTATTTCTGTGCAAGAGAGTGGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGC
TAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACA
GCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAG
GCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCT
CAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAAT
CACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACA
CATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAA
ACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC
CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGA
CAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCA
CCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCC
ATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCC
CATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCC
CAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCT
CCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGT
GGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCA
GAAGAGCCTCTCCCTGTCTCCGGGTAAAAGTTCATCACCAGATAAACAAATGGCAGTGCTTCCT
AGAAGAGAGCGGAATCGGCAGGCTGCAGCTGCCAACCCAGAGAATTCCAGAGGAAAAGGTCGGA
GAGGCCAGAGGGGCAAAAACCGGGGTTGTGTCTTAACTGCAATACATTTAAATGTCACTGACTT
GGGTCTGGGCTATGAAACCAAGGAGGAACTGATTTTTAGGTACTGCAGCGGCTCTTGCGATGCA
GCTGAGACAACGTACGACAAAATATTGAAAAACTTATCCAGAAATAGAAGGCTGGTGAGTGACA
AAGTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGATGATGACCTGTCGTTTTTAGATGATAA
CCTGGTTTACCATATTCTAAGAAAGCATTCCGCTAAAAGGTGTGGATGTATCTGAAACCCGAGC
TCGGTACCAAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCAT
CTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTC
CTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG
GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGG
GCTCTATGGCTTCTGAGGCGGAAAGAACCAGGGGAGGTACCGAGCTCTTACGCGTGCTAGCTCG
AGATCTGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTA
ACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGG
CCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGG
CTTTTGCAAAAAGCTTATCGATTCTAGAAGCCGCCACCATGGTTCGACCATTGAACTGCATCGT
CGCCGTGTCCCAAAATATGGGGATTGGCAAGAACGGAGACCTACCCTGGCCTCCGCTCAGGAAC
GAGTTCAAGTACTTCCAAAGAATGACCACAACCTCTTCAGTGGAAGGTAAACAGAATCTGGTGA
TTATGGGTAGGAAAACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGACAGAATTAA
TATAGTTCTCAGTAGAGAACTCAAAGAACCACCACGAGGAGCTCATTTTCTTGCCAAAAGTTTG
GATGATGCCTTAAGACTTATTGAACAACCGGAATTGGCAAGTAAAGTAGACATGGTTTGGATAG
TCGGAGGCAGTTCTGTTTACCAGGAAGCCATGAATCAACCAGGCCACCTCAGACTCTTTGTGAC
AAGGATCATGCAGGAATTTGAAAGTGACACGTTTTTCCCAGAAATTGATTTGGGGAAATATAAA
CTTCTCCCAGAATACCCAGGCGTCCTCTCTGAGGTCCAGGAGGAAAAAGGCATCAAGTATAAGT
TTGAAGTCTACGAGAAGAAAGACTAACAGGAAGATGCTTTCAAGTTCTCTGCTCCCTCCTAAA
GCTATGCATTTTTATAAGACCATGGGACTTTTGCTGGCTTTAGATCCTTCGCGGGACGTCCTTT
GTTTACGTCCCGTCGGCGCTGAATCCCGCGGACGACCCCTCGCGGGGCCGCTTGGGACTCTCTC
GTCCCCTTCTCCGTCTGCCGTTCCAGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCC
GTCTGTGCCTTCTCATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGCACGTTGCATGGAGA
CCACCGTGAACGCCCATCAGATCCTGCCCAAGGTCTTACATAAGAGGACTCTTGGACTCCCAGC
AATGTCAACGACCGACCTTGAGGCCTACTTCAAAGACTGTGTGTTTAAGGACTGGGAGGAGCTG
GGGGAGGAGATTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGGTCTGCGCAC
CAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTGTTCAAGCC
TCCAAGCTGTGCCTTGGGTGGCTTTGGGGCATGGACATTGACCCTTATAAAGAATTTGGAGCTA
CTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTCCTTCCGTCAGAGATCCTCTACGCCG
GACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACAT

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CACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATG
GTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGG
CGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGG
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<210>      48
<211>      234
<212>      PRT
<213>      artificial sequence

<220>
<223>      Description of Artificial Sequence: Synthetic Protein

<400>      48
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TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
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<210>      49
<211>      598
<212>      PRT
<213>      artificial sequence

<220>
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GDGSTKYNEKFKGKATLTADKSSSTAYMHLSSLTSEKSAVYFCAREWAYWGQGTLVTVSAASTKGPSVFPLA
PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGKSSSPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRG
CVLTAIHLNVTDLGLGYETKEELIFRYCSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLS
FLDDNLVYHILRKHSAKRCGCI
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<210>      50
<211>      187
<212>      PRT
<213>      artificial sequence

<220>
<223>      Description of Artificial Sequence: Synthetic Protein

<400>      50
MVRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLVIMGRKTWFSIPEKNRPLKDRI
NIVLSRELKEPPRGAHFLAKSLDDALRLIEQPELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFE
SDTFFPEIDLGKYKLLPEYPGVLSEVQEEKGIKYKFEVYEKKD
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<210> 51
<211> 405
<212> DNA
<213> artificial sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 51
TCACCAGATAAACAAATGGCAGTGCTTCCTAGAAGAGAGCGGAATCGGCAGGCTGCAGCTGCCAACCCAGAG
AATTCCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGTTGTGTCTTAACTGCAATACATTTA
AATGTCACTGACTTGGGTCTGGGCTATGAAACCAAGGAGGAACTGATTTTTAGGTACTGCAGCGGCTCTTGC
GATGCAGCTGAGACAACGTACGACAAAATATTGAAAAACTTATCCAGAAATAGAAGGCTGGTGAGTGACAAA
GTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGATGATGACCTGTCGTTTTTAGATGATAACCTGGTTTAC
CATATTCTAAGAAAGCATTCCGCTAAAAGGTGTGGATGTATCTGA

<210> 52
<211> 134
<212> PRT
<213> artificial sequence

<220>
<223> Description of Artificial Sequence: Synthetic Protein

<400> 52
SPDKQMAVLPRRERNRQAAAANPENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYETKEELIFRYCSGSC
DAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

CLAIMS

WHAT IS CLAIMED IS:

1.   A composition comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52) covalently linked to a structure that is capable of crossing the blood brain barrier (BBB).

2.   The composition of claim 1 wherein the structure that is capable of crossing the BBB crosses the BBB on an endogenous BBB receptor.

3.   The composition of claim 2 wherein the endogenous BBB receptor is selected from the group consisting of the insulin receptor, transferrin receptor, leptin receptor, lipoprotein receptor, and the IGF receptor.

4.   The composition of claim 3 wherein the endogenous BBB receptor mediated transport system is the insulin BBB receptor mediated transport system.

5.   The composition of claim 1 wherein the structure that is capable of crossing the BBB is an antibody.

6.   The composition of claim 5 wherein the antibody is a monoclonal antibody (MAb).

7.   The composition of claim 6 wherein the MAb is a chimeric MAb.

8.   The composition of claim 2 wherein the structure that crosses the BBB on an endogenous BBB receptor mediated transport system is an antibody.

9.   The composition of claim 8 wherein the antibody is a monoclonal antibody.

10.   The composition of claim 9 wherein the MAb is a chimeric MAb.

11.   The composition of claim 10.wherein the chimeric antibody contains at least about 80% human sequence.

12.   The composition of claim 1, wherein the neurotherapeutic peptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52).

13.   The composition of claim 12, wherein the neurotherapeutic peptide comprises the amino acid sequence of mature human GDNF (SEQ ID NO:52).

14.   The composition of claim 6, wherein the neurotherapeutic peptide is covalently linked at its amino terminus to the carboxy terminus of the heavy chain of the MAb.

15.   A composition for treating a neurological disorder comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52) covalently linked to an immunoglobulin that is capable of crossing the blood brain barrier, wherein the composition is capable of crossing the BBB in an amount that is effective in treating the neurological disorder.

16.   A recombinant mammalian cell comprising the composition of claim 15.

17.   A method for treating a CNS disorder in an individual comprising peripherally administering to the individual an effective amount of a composition comprising a neurotherapeutic peptide

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comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52) covalently attached to a structure capable of crossing the BBB.

18.   The method of claim 17, wherein the neurotherapeutic peptide comprises an amino acid sequence at least 95% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52).

19.   The method of claim 18, wherein the neurotherapeutic peptide comprises the amino acid sequence of mature human GDNF (SEQ ID NO:52).

20.   The method of claim 17 wherein the administering is selected from the group consisting of oral, intravenous, intramuscular, subcutaneous, intraperitoneal, rectal, transbuccal, intranasal, transdermal, and inhalation administration.

21.   The method of claim 20 wherein the administering is intravenous, intramuscular, or subcutaneous.

22.   The method of claim 17 wherein the CNS disorder is an acute CNS disorder.

23.   The method of claim 22 wherein the acute CNS disorder is selected from the group consisting of spinal cord injury, brain injury, focal brain ischemia and global brain ischemia.

24.   The method of claim 17 wherein the CNS disorder is a chronic disorder.

25.   The method of claim 24 wherein the chronic disorder is selected from the group consisting of chronic neurodegenerative disease, drug addiction, or alcohol addiction.

26.   The method of claim 25 wherein the chronic neurodegenerative disease is selected from the group consisting of Parkinson's disease or a motor neuron disease.

27.   The method of claim 17 wherein the individual is administered a dose of the composition that is about 1 to about 100 mg.

28.   A method for treating substance abuse in an individual, comprising administering to the individual an effective amount of a composition comprising a neurotherapeutic peptide comprising an amino acid sequence which is at least 80% identical to the amino acid sequence of mature human GDNF (SEQ ID NO:52) covalently attached to a structure capable of crossing the BBB.

FIG. 1

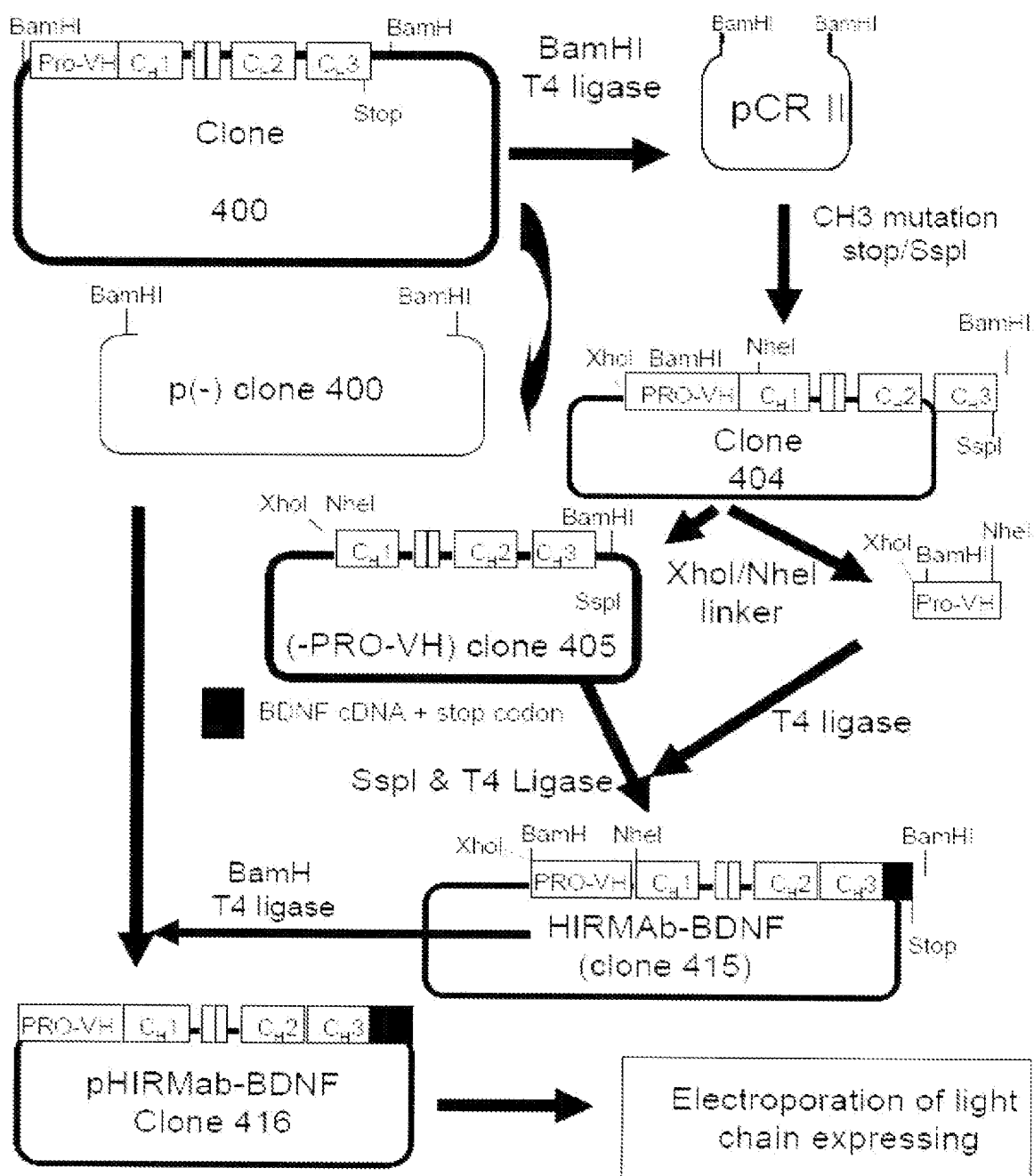Engineering of intron-based expression vector for fusion protein heavy chain
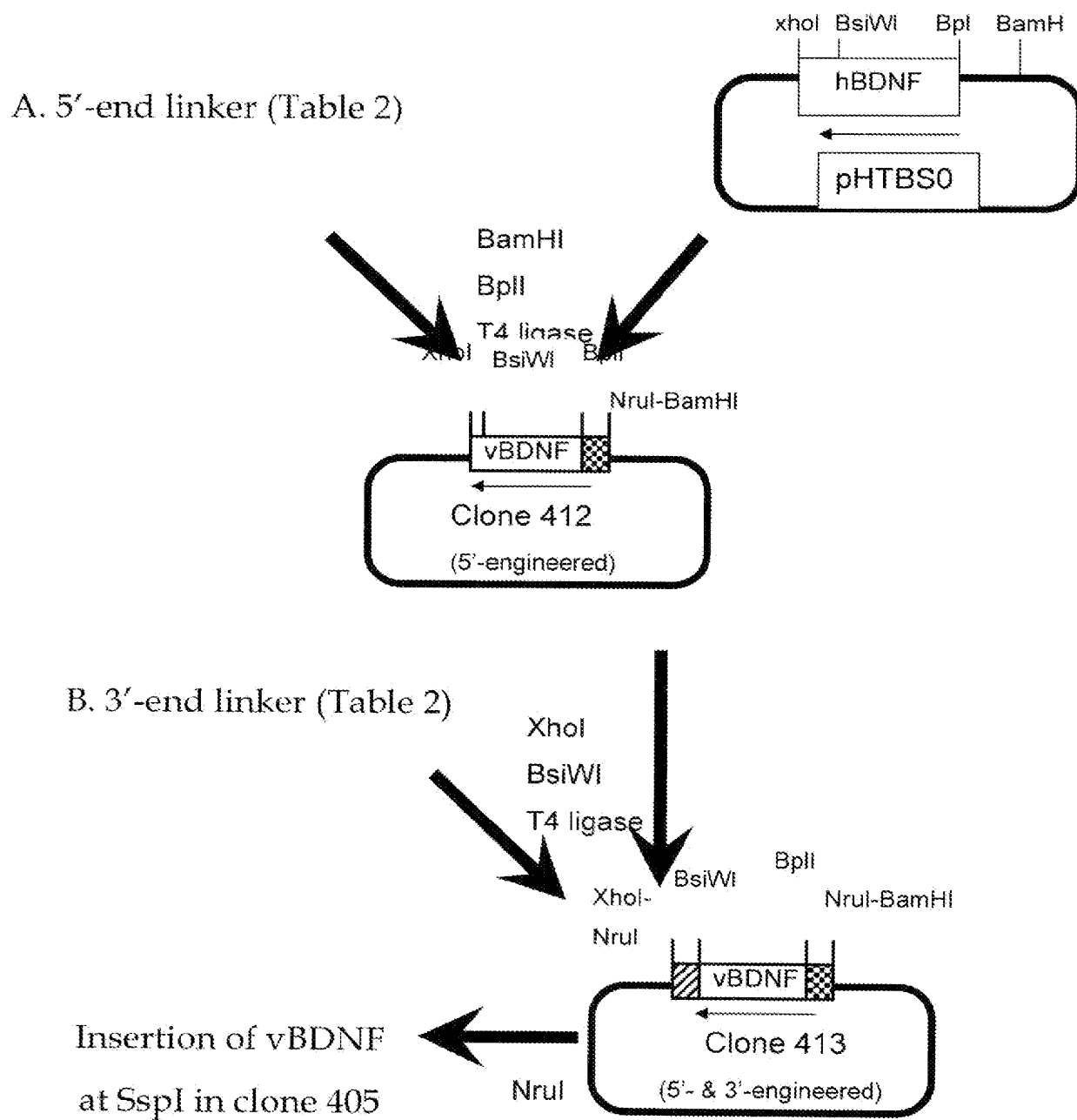
FIG. 2
Engineering of vBDNF

A. 5′-end linker (Table 2)
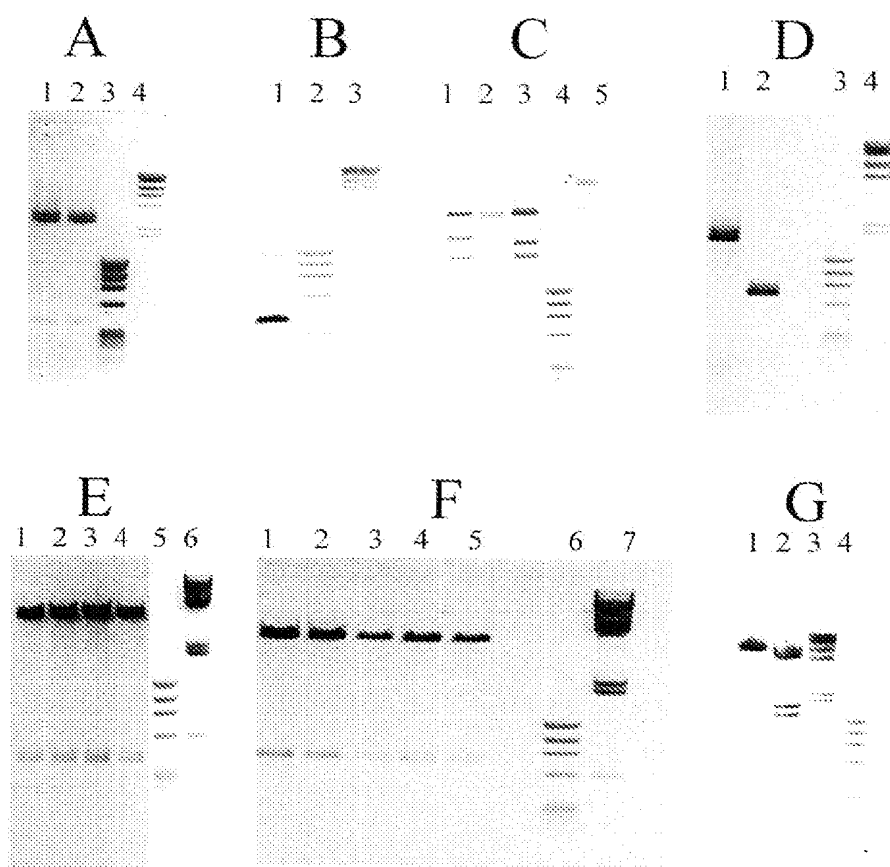
xhoI BsiWI   BpI   BamH

hBDNF

pHTBS0

BamHI

BpII

T4 ligase
XhoI   BsiWI   BpI

Nrul-BamHI

vBDNF

Clone 412

(5′-engineered)

B. 3′-end linker (Table 2)

XhoI

BsiWI

T4 ligase
XhoI-   BsiWI   BpII
Nrul   Nrul-BamHI

vBDNF

Insertion of vBDNF   Clone 413
at SspI in clone 405   Nrul   (5′- & 3′-engineered)

FIG. 3
Agarose gel electrophoresis

FIG. 4
Engineered cDNA and amino acid sequence corresponding to the end of CH3,
CH3-vBDNF linker and vBDNF, of the fusion protein
(SEQ ID NO. 21 and 22)

```
CTG TCT CCG GGT AAA tcg agt atg cac tct gac cct gcc cgt cga ggt
gag ctg agc gtg
leu ser pro gly lys ser ser met his ser asp pro ala arg arg gly
glu leu ser val
      CH3 orf end              linker  BDNF orf
                                       _____→


tgt gac agt att agt gag tgg gta acg gcg gca gac aaa aag act gca
gtg gac atg tcg
cys asp ser ile ser glu trp val thr ala ala asp lys lys thr ala
val asp met ser


ggc ggg acg gtc aca gtc ctt gaa aag gtc cct gta tca aaa ggc caa
ctg aag caa tac
gly gly thr val thr val leu glu lys val pro val ser lys gly gln
leu lys gln tyr


ttc tac gag acc aag tgc aat ccc atg ggt tac aca aaa gaa ggc tgc
agg ggc ata gac
phe tyr glu thr lys cys asn pro met gly tyr thr lys glu gly cys
arg gly ile asp


aaa agg cat tgg aac tcc cag tgc cga act acc cag tcg tac gtg cgg
gcc ctt acc atg
lys arg his trp asn ser gln cys arg thr thr gln ser tyr val arg
ala leu thr met


gat agc aaa aag aga att ggc tgg cga ttc ata agg ata gac act tct
tgt gta tgt aca
asp ser lys lys arg ile gly trp arg phe ile arg ile asp thr ser
cys val cys thr


ttg acc att aaa agg tga
leu thr ile lys arg  *
```

FIG. 5A

Nucleotide Sequence of Fusion Protein Heavy Chain Gene Derived from Clone 416(2711nt)
(SEQ ID NO. 23)

TAGTCTTTCTCTTCAGTGACAAACACAGACATAGGATATTCCACC<u>ATG</u>GAATGCAGCTGGGTCATGCTCT

TCCTCCTGTCAGGAACTGCAGGTGTCCATTGCCAGGTTCAGCTGCAGCAGTCTGGACCTGAGCTGGTGAA

GCCTGGGGCTTTAGTGAAGATATCCTGCAAGGCTTCTGGTTACACCTTCACAAACTACGATATACACTGG

GTGAAGCAGAGGCCTGGACAGGGACTTGAGTGGATTGGATGGATTTATCCTGGAGATGGTAGTACTAAGT

ACAATGAGAAATTCAAGGGCAAGGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCACCT

CAGCAGCCTGACTTCTGAGAAATCTGCAGTCTATTTCTGTGCAAGAGAGTGGGCTTACTGGGGCCAAGGG

ACTCTGGTCACTGTCTCTGCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGA

GCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTT**CCCCGAACCGGTGACGGTGTC**

GTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTAC

TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATC

ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTT*GGTGAGAGGCCAGCACAGGGAGGGAGGGTGTCTGC*

TGGAAGCCAGGCTCAGCGCTCCTGCCTGGACGCATCCCGGCTATGCAGCCCCAGTCCAGGGCAGCAAGGC

AGGCCCCGTCTGCCTCTTCACCCGGAGGCCTCTGCCCGCCCCACTCATGCTCAGGGAGAGGGTCTTCTGG

CTTTTTCCCCAGGCTCTGGGCAGGCACAGGCTAGGTGCCCCTAACCCAGGCCCTGCACACAAAGGGGCAG

GTGCTGGGCTCAGACCTGCCAAGAGCCATATCCGGGAGGACCCTGCCCCTGACCTAAGCCCACCCCAAAG

GCCAAACTCTCCACTCCCTCAGCTCGGACACCTTCTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTC

*TCTGCAG***AGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCA***GGTAAGCCAGCCCAGGCCT*

CGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCCAGGGACAGGCCCCAGCCGGGTG

*CTGACACGTCCACCTCCATCTCTTCCTCAG***CACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCC**

CCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC

CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGC

CGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGGGTCCTCACCGTCCTGCACCAGGACTGGCT

GAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCC

AAAGCCAAAG*GTGGGACCCGTGGGGTGCGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACCCTCTGCC*

*CTGAGAGTGACCGCTGTACCAACCTCTGTCCCTACAG***GGCAGCCCCGAGAACCACAGGTGTACACCCTGC**

CCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAG

CGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTG

GACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAG

FIG. 5B

Nucleotide Sequence of Fusion Protein Heavy Chain Gene Derived from Clone
416(2711nt)
(SEQ ID NO. 23 con't)

GTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACG

CAGAAGAGCCTCTCCCTGTCTCCGGGTAAATCGAGTATG*CACTCTGACCCTGCCCGTCGAGGTG*

AGCTGAGCGTGTGTGACAGTATTAGTGAGTGGGTAACGGCGGCAGACAAAAAGACTGCAGTGGA

CATGTCGGGCGGGACGGTCACAGTCCTTGAAAAGGTCCCTGTATCAAAAGGCCAACTGAAGCAA

TACTTCTACGAGACCAAGTGCAATCCCATGGGTTACACAAAAGAAGGCTGCAGGGGCATAGACA

AAAGGCATTGGAACTCCCAGTGCCGAACTACCCAGTCGTACGTGCGGGCCCTTACCATGGATAG

CAAAAAGAGAATTGGCTGGCGATTCATAAGGATAGACACTTCTTGTGTATGTACATTGACCATT

*AAAAGG**TGA**TCGATTTTGCGACGGCCGGCAAGCCCCCGCTCCCCGGGCTCTCGCGGTCGCACGA*

GGATGCTTGGCACGTACCCCCTGTACATACTTCCCGGGCGCCCAGCATGGAAATAAAGCACCCA

GCGCTGCCCTGGGCCCCTGCGAGACTGTGATGGTTCTTTCCACGGGTCAGGCCGAGTCTGAGGC

CTGAGTGGCATGAGGGAGGCAGAGCGGGTCCCACTGTCCCCACACTGGCCCAGGCTGTGCAGGT

GTGCCTGGGCCGCCTAGGGTGGGGCTCAGCCAGGGGCTGCCCTCGGCAGGGTGGGGGATTTGCC

FIG. 6

Amino Acid Sequence of Heavy Chain of Fusion Protein

(SEQ ID NO. 24)

NH2 -

MECSWVMLFLLSGTAGVHCQVQLQQSGPELVKPGALVKISCKASGYTFTNYDIHW

VKQRPGQGLEWIGWIYPGDGSTKYNEKFKGKATLTADKSSSTAYMHLSSLTSEKS

AVYFCAREWAYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF

PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK

PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT

CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**NST**YRVVRVLTVLHQDWLN

GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK

GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC

SVMHEALHNHYTQKSLSLSPGKSSMHSDPARRGELSVCDSISEWVTAADKKTAVD

MSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCRTTQS

YVRALTMDSKKRIGWRFIRIDTSCVCTLTIKR - COOH

FIG. 7
Amino acid sequence of the fusion protein heavy chain
(SEQ. ID NO. 25)

Signal peptide:

MECSWVMLFLLSGTAGVHC

FR1:

QVQLQQSGPELVKPGALVKISCKAS

CDR1:

GYTFTNYDIH

FR2:

WVKQRPGQGLEWIG

CDR2:

WIYPGDGSTKYNEKFKG

FR3:

KATLTADKSSSTAYMHLSSLTSEKSAVYFCAR

CDR3:

EWAY

FR4:

WGQGTLVTVSA

CH1:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT

VPSSSLGTQTYICNVNHKPSNTKVDKKV

Hinge:

EPKSCDKTHTCP

CH2:

PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN

STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK

CH3:

GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL

TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

linker:

SSM

vBDNF:

HSDPARRGELSVCDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRG

IDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKR

FIG. 8

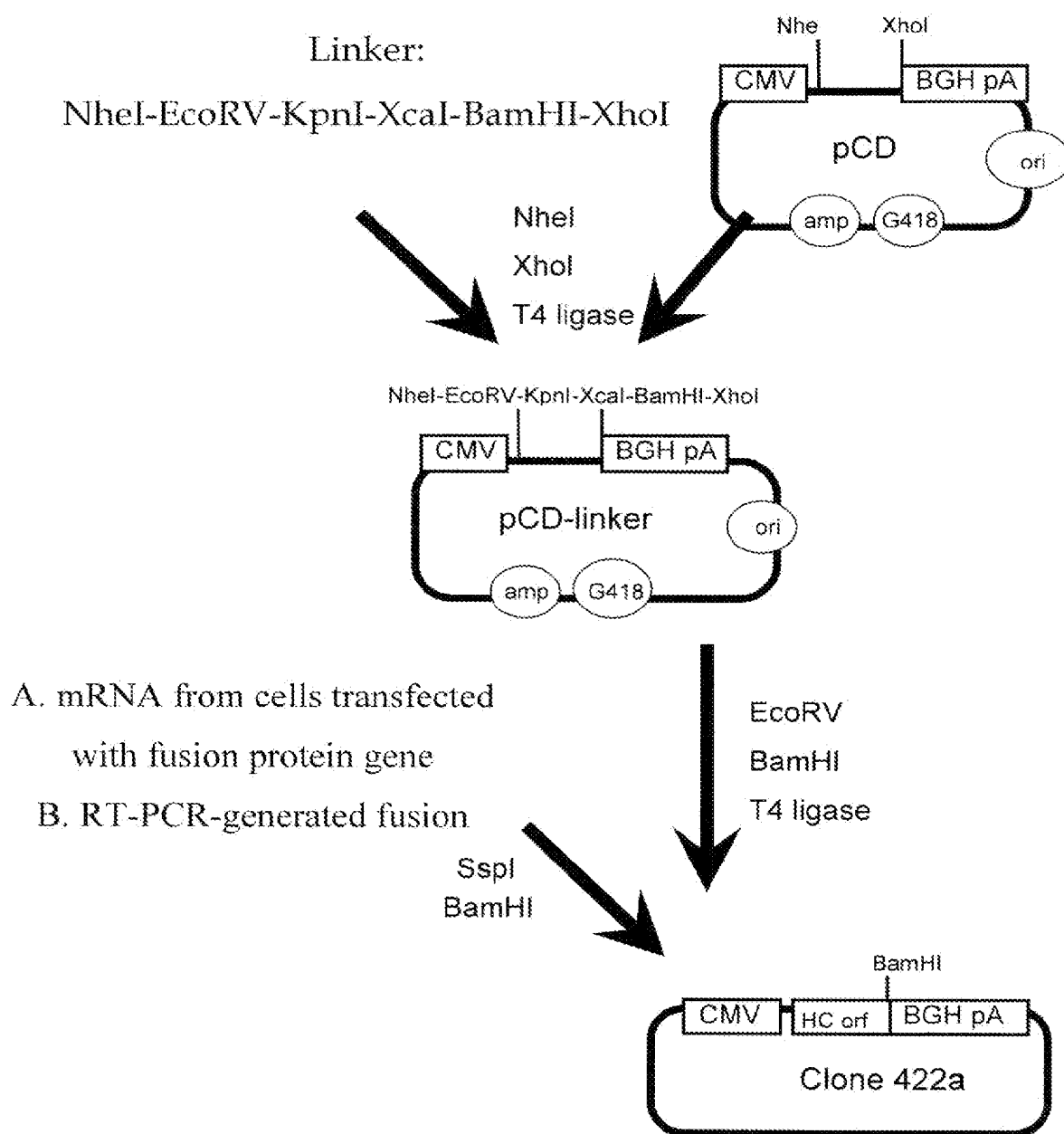Engineering of intronless pCD-HC-120 expression vector

FIG. 9A

Nucleotide sequence of fusion protein heavy chain cDNA in clone 422a
(SEQ ID NO. 26)

ATTCCACCATGGAATGCAGCTGGGTCATGCTCTTCCTCCTGTCAGGAACTGCAGGTGTCCATTGCCAGGT
TCAGCTGCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTTAGTGAAGATATCCTGCAAGGCTTCT
GGTTACACCTTCACAAACTACGATATACACTGGGTGAAGCAGAGGCCTGGACAGGGACTTGAGTGGATTG
GATGGATTTATCCTGGAGATGGTAGTACTAAGTACAATGAGAAATTCAAGGGCAAGGCCACACTGACTGC
AGACAAATCCTCCAGCACAGCCTACATGCACCTCAGCAGCCTGACTTCTGAGAAATCTGCAGTCTATTTC
TGTGCAAGAGAGTGGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCTAGCACCAAGGGCC
CATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGT
CAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACC
TTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCT
TGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGA
GCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCA
GTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGG
TGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAA
TGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTG
CACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCG
AGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGA
TGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG
GAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCT
CCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTC
CGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATCGAGT
ATGCACTCTGACCCTGCCCGTCGAGGTGAGCTGAGCGTGTGTGACAGTATTAGTGAGTGGGTAACGGCGG
CAGACAAAAAGACTGCAGTGGACATGTCGGGCGGGACGGTCACAGTCCTTGAAAAGGTCCCTGTATCAAA
AGGCCAACTGAAGCAATACTTCTACGAGACCAAGTGCAATCCCATGGGTTACACAAAAGAAGGCTGCAGG
GGCATAGACAAAAGGCATTGGAACTCCCAGTGCCGAACTACCCAGTCGTACGTGCGGGCCCTTACCATGG
ATAGCAAAAAGAGAATTGGCTGGCGATTCATAAGGATAGACACTTCTTGTGTATGTACATTGACCATTAA
AAGGTGA

ATT: ½ SspI

CCACC: Kozak

FIG. 9B

Deduced amino acid sequence of fusion protein heavy chain cDNA in clone 422a
(SEQ ID NO. 27)

```
Atggaatgcagctgggtcatgctcttcctcctgtcaggaactgca
 M   E   C   S   W   V   M   L   F   L   L   S   G   T   A
ggtgtccattgccaggtcagctgcagcagtctggacctgagctg
 G   V   H   C   Q   V   Q   L   Q   Q   S   G   P   E   L
gtgaagcctgggcttagtgaagatatcctgcaaggcttctggt
 V   K   P   G   A   L   V   K   I   S   C   K   A   S   G
tacaccttcacaaactacgatatacactgggtgaagcagaggcct
 Y   T   F   T   N   Y   D   I   H   W   V   K   Q   R   P
ggacagggacttgagtggattggatggatttatcctggagatggt
 G   Q   G   L   E   W   I   G   W   I   Y   P   G   D   G
agtactaagtacaatgagaaattcaaggcaaggccacactgact
 S   T   K   Y   N   E   K   F   K   G   K   A   T   L   T
gcagacaaatcctccagcacagcctacatgcacctcagcagcctg
 A   D   K   S   S   S   T   A   Y   M   H   L   S   S   L
acttctgagaaatctgcagtctatttctgtgcaagagagtgggct
 T   S   E   K   S   A   V   Y   F   C   A   R   E   W   A
tactggggccaagggactctggtcactgtctctgcagctagcacc
 Y   W   G   Q   G   T   L   V   T   V   S   A   A   S   T
aagggcccatcggtcttccccctggcaccctcctccaagagcacc
 K   G   P   S   V   F   P   L   A   P   S   S   K   S   T
tctgggggcacagcggccctgggctgcctggtcaaggactacttc
 S   G   G   T   A   A   L   G   C   L   V   K   D   Y   F
cccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagc
 P   E   P   V   T   V   S   W   N   S   G   A   L   T   S
ggcgtgcacaccttcccggctgtcctacagtcctcaggactctac
 G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y
tccctcagcagcgtggtgaccgtgccctccagcagcttgggcacc
 S   L   S   S   V   V   T   V   P   S   S   L   G   T
cagacctacatctgcaacgtgaatcacaagcccagcaacaccaag
 Q   T   Y   I   C   N   V   N   H   K   P   S   N   T   K
gtggacaagaaagttgagcccaaatcttgtgacaaaactcacaca
 V   D   K   K   V   E   P   K   S   C   D   K   T   H   T
tgcccaccgtgcccagcacctgaactcctggggggaccgtcagtc
 C   P   P   C   P   A   P   E   L   L   G   G   P   S   V
ttcctcttccccccaaaacccaaggacacccteatgatctcccgg
 F   L   F   P   P   K   P   K   D   T   L   M   I   S   R
acccctgaggtcacatgcgtggtggtggacgtgagccacgaagac
 T   P   E   V   T   C   V   V   V   D   V   S   H   E   D
cctgaggtcaagttcaactggtacgtggacggcgtggaggtgcat
```

FIG. 9C

Deduced amino acid sequence of fusion protein heavy chain cDNA in clone 422a
(SEQ ID NO. 28)

```
        P   E   V   K   F   N   W   Y   V   D   G   V   E   V   H
        aatgccaagacaaagccgcgggaggagcagtacaacagcacgtac
        N   A   K   T   K   P   R   E   E   Q   Y   N   S   T   Y
        cgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaat
        R   V   V   S   V   L   T   V   L   H   Q   D   W   L   N
        ggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcc
        G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A
        cccatcgagaaaaccatctccaaagccaaagggcagccccgagaa
        P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E
        ccacaggtgtacaccctgcccccatcccgggatgagctgaccaag
        P   Q   V   Y   T   L   P   P   S   R   D   E   L   T   K
        aaccaggtcagcctgacctgcctggtcaaaggcttctatcccagc
        N   Q   V   S   L   T   C   L   V   K   G   F   Y   P   S
        gacatcgccgtggagtgggagagcaatgggcagccggagaacaac
        D   I   A   V   E   W   E   S   N   G   Q   P   E   N   N
        tacaagaccacgcctcccgtgctggactccgacggctccttcttc
        Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F
        ctctacagcaagctcaccgtggacaagagcaggtggcagcagggg
        L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G
        aacgtcttctcatgctccgtgatgcatgaggctctgcacaaccac
        N   V   F   S   C   S   V   M   H   E   A   L   H   N   H
        tacacgcagaagagcctctccctgtctccgggtaaatcgagtatg
        Y   T   Q   K   S   L   S   L   S   P   G   K   S   S   M
        cactctgaccctgcccgtcgaggtgagctgagcgtgtgtgacagt
        H   S   D   P   A   R   R   G   E   L   S   V   C   D   S
        attagtgagtgggtaacggcggcagacaaaaagactgcagtggac
        I   S   E   W   V   T   A   A   D   K   K   T   A   V   D
        atgtcgggcgggacggtcacagtccttgaaaaggtccctgtatca
        M   S   G   G   T   V   T   V   L   E   K   V   P   V   S
        aaaggccaactgaagcaatacttctacgagaccaagtgcaatccc
        K   G   Q   L   K   Q   Y   F   Y   E   T   K   C   N   P
        atgggttacacaaaagaaggctgcagggggcatagacaaaaggcat
        M   G   Y   T   K   E   G   C   R   G   I   D   K   R   H
        tggaactcccagtgccgaactacccagtcgtacgtgcgggccctt
        W   N   S   Q   C   R   T   T   Q   S   Y   V   R   A   L
        accatggatagcaaaaagagaattggctggcgattcataaggata
        T   M   D   S   K   K   R   I   G   W   R   F   I   R   I
        gacacttcttgtgtatgtacattgaccattaaaaggtga 1757
        D   T   S   C   V   C   T   L   T   I   K   R   *
```
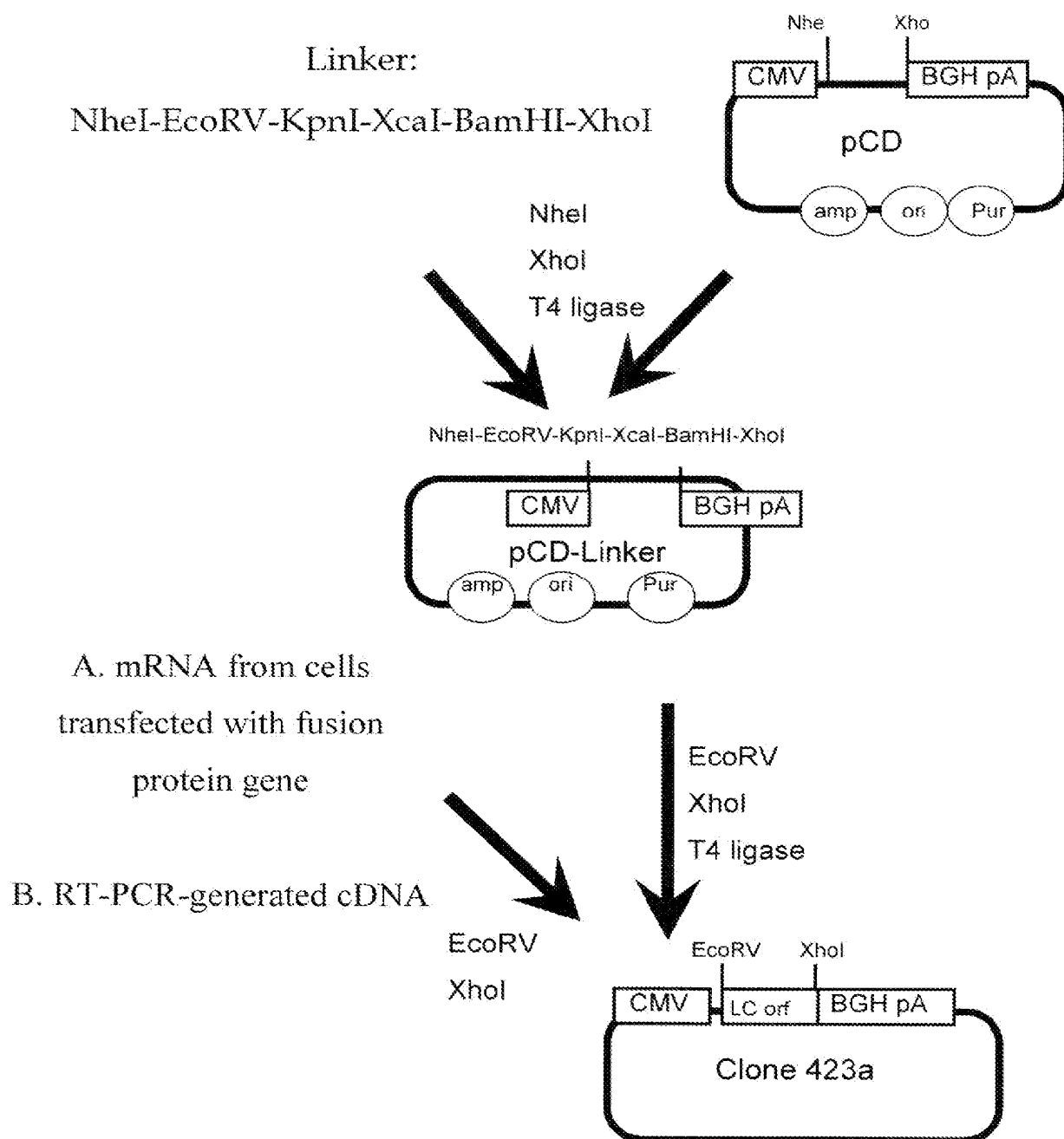
FIG. 10

Engineering of intronless pCD-LC-1 expression vector

Linker:

NheI-EcoRV-KpnI-XcaI-BamHI-XhoI

NheI
XhoI
T4 ligase

Nhe   Xho
CMV   BGH pA
pCD
amp ori Pur

NheI-EcoRV-KpnI-Xcal-BamHI-Xhol
CMV   BGH pA
pCD-Linker
amp ori Pur

A. mRNA from cells transfected with fusion protein gene

B. RT-PCR-generated cDNA

EcoRV
XhoI
T4 ligase

EcoRV
XhoI

EcoRV   Xhol
CMV   LC orf   BGH pA
Clone 423a

FIG. 11A

Nucleotide sequence of fusion protein light chain cDNA in clone 423a

(SEQ ID NO. 29)

GATATCACCATGGAGACAGACACACTCCTGCTATGGCTCTTGTTGCTCATGTTTCCA
GGTACCAGATGTGACATCCAGATGACCCAGTCTCCATCCTCCTTATCTGCCTCTCTG
GGAGAAAGAGTCAGTCTCACTTGTCGGGCAAGTCAGGACATTGGTGGTAACTTATA
CTGGCTTCAGCAGGGACCAGATGGAACTATTAAACGCCTGATCTACGCCACATCCA
GTTTAGATTCTGGTGTCCCCAAAAGGTTCAGTGGCAGTAGGTCTGGGTCAGATTATT
CTCTCACCATCAGCAGCCTTGAGTCTGAAGATTTTGTAGACTATTACTGTCTACAGTA
TTCTAGTTCTCCGTGGACGTTCGGTGGAGCGACAAAGATGGAAATAAAACGAACTG
TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGT
GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAG
GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGA
CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGC
CCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG

GATATC: EcoR V

CACC: Kozak

FIG. 11B

Deduced amino acid sequence of fusion protein light chain in clone 423a

(SEQ ID NO. 30 and 31)

```
atggagacagacacactcctgctatggctcttgttgctcatgttt
 M   E   T   D   T   L   L   L   W   L   L   L   L   M   F
ccaggtaccagatgtgacatccagatgacccagtctccatcctcc
 P   G   T   R   C   D   I   Q   M   T   Q   S   P   S   S
ttatctgcctctctgggagaaagagtcagtctcacttgtcgggca
 L   S   A   S   L   G   E   R   V   S   L   T   C   R   A
agtcaggacattggtggtaacttatactggcttcagcagggacca
 S   Q   D   I   G   G   N   L   Y   W   L   Q   Q   G   P
gatggaactattaaacgcctgatctacgccacatccagtttagat
 D   G   T   I   K   R   L   I   Y   A   T   S   S   L   D
tctggtgtccccaaaaggttcagtggcagtaggtctgggtcagat
 S   G   V   P   K   R   F   S   G   S   R   S   G   S   D
tattctctcaccatcagcagccttgagtctgaagattttgtagac
 Y   S   L   T   I   S   S   L   E   S   E   D   F   V   D
tattactgtctacagtattctagttctccgtggacgttcggtgga
 Y   Y   C   L   Q   Y   S   S   P   W   T   F   G   G
gcgacaaagatggaaataaaacgaactgtggctgcaccatctgtc
 A   T   K   M   E   I   K   R   T   V   A   A   P   S   V
ttcatcttcccgccatctgatgagcagttgaaatctggaactgcc
 F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A
tctgttgtgtgcctgctgaataacttctatcccagagaggccaaa
 S   V   V   C   L   N   N   F   Y   P   R   E   A   K
gtacagtggaaggtggataacgccctccaatcgggtaactcccag
 V   Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q
gagagtgtcacagagcaggacagcaaggacagcacctacagcctc
 E   S   V   T   E   Q   D   S   K   D   S   T   Y   S   L
agcagcaccctgacgctgagcaaagcagactacgagaaacacaaa
 S   S   T   L   T   L   S   K   A   D   Y   E   K   H   K
gtctacgcctgcgaagtcacccatcagggcctgagctcgcccgtc
 V   Y   A   C   E   V   T   H   Q   G   L   S   S   P   V
acaaagagcttcaacaggggagagtgttag
 T   K   S   F   N   R   G   E   C   *
```

FIG. 12

Engineering of fusion protein tandem expression vector

FIG. 13A

Nucleotide sequence of the fusion protein heavy chain (HC) and light chain (LC) genes, and the
DHFR gene in the tandem vector
(SEQ ID NO. 32)

HC gene (forward)

CGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCAT

TAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAAC

GACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA

ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTA

TTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGG

CAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATA

GCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATC

AACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAG

GTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA

CTATAGGGAGACCCTTGCTAGCGATATTCCACCATGGAATGCAGCTGGGTCATGCTCTTCCTCCTGTCAGGAAC

TGCAGGTGTCCATTGCCAGGTTCAGCTGCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTTAGTGAAGA

TATCCTGCAAGGCTTCTGGTTACACCTTCACAAACTACGATATACACTGGGTGAAGCAGAGGCCTGGACAGGGA

CTTGAGTGGATTGGATGGATTTATCCTGGAGATGGTAGTACTAAGTACAATGAGAAATTCAAGGGCAAGGCCAC

ACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCACCTCAGCAGCCTGACTTCTGAGAAATCTGCAGTCT

ATTTCTGTGCAAGAGAGTGGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCTAGCACCAAGGGC

CCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA

GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGG

CTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAG

ACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGA

CAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAA

AACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGAC

CCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCA

GTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACA

AGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGA

GAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGT

CAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCA

CGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAG

CAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCT

GTCTCCGGGTAAATCGAGTATGCACTCTGACCCTGCCCGTCGAGGTGAGCTGAGCGTGTGTGACAGTATTAGTG

FIG. 13B

AGTGGGTAACGGCGGCAGACAAAAAGACTGCAGTGGACATGTCGGGCGGGACGGTCACAGTCCTTGAAAAGGTC

CCTGTATCAAAAGGCCAACTGAAGCAATACTTCTACGAGACCAAGTGCAATCCCATGGGTTACACAAAAGAAGG

CTGCAGGGGCATAGACAAAAGGCATTGGAACTCCCAGTGCCGAACTACCCAGTCGTACGTGCGGGCCCTTACCA

TGGATAGCAAAAAGAGAATTGGCTGGCGATTCATAAGGATAGACACTTCTTGTGTATGTACATTGACCATTAAA

AGGTGAGGATCCCTCGAGCATGCATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGAT

CAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAA

GGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTAT

TCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGG

TGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGTGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGC

AGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACC

LC gene (forward)

GAATTCGATATTCCATACACATACTTCTGTGTTCCTTTGAAAGCTGGACTTTTGCAGGCTCCACCAGACCTCTC

TAGATCAATTCCTTTGCCTAATTTCGCTTACAATTTACGCGCGCGTTGACATTGATTATTGACTAGTTATTAAT

AGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC

CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAAT

AGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATC

ATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACC

TTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCA

GTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGA

GTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGC

GGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCT

TATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGATATCACCATGGAGACAGACACACTC

CTGCTATGGCTCTTGTTGCTCATGTTTCCAGGTACCAGATGTGACATCCAGATGACCCAGTCTCCATCCTCCTT

ATCTGCCTCTCTGGGAGAAAGAGTCAGTCTCACTTGTCGGGCAAGTCAGGACATTGGTGGTAACTTATACTGGC

TTCAGCAGGGACCAGATGGAACTATTAAACGCCTGATCTACGCCACATCCAGTTTAGATTCTGGTGTCCCCAAA

AGGTTCAGTGGCAGTAGGTCTGGGTCAGATTATTCTCTCACCATCAGCAGCCTTGAGTCTGAAGATTTTGTAGA

CTATTACTGTCTACAGTATTCTAGTTCTCCGTGGACGTTCGGTGGAGCGACAAAGATGGAAATAAAACGAACTG

TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGC

CTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTC

CCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAG

CAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGC

FIG. 13C

TTCAACAGGGGAGAGTGTTAGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTC

TAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAG

GTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATT

CTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGT

GGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGTGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGAA

ATGAGGACTTAACCTGTGGAAATATCAAGCTT

LC gene-DHFR gene linker

GCGGCCGCGTA

DHFR gene (reverse)

TCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCG

CCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACG

CCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCC

AGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCTCTGACGGAAGGAAAG

AAGTCAGAAGGCAAAAACGAGAGTAACTCCACAGTAGCTCCAAATTCTTTATAAGGGTCAATGTCCATGCCCCA

AAGCCACCCAAGGCACAGCTTGGAGGCTTGAACAGTGGGACATGTACAAGAGATGATTAGGCAGAGGTGAAAAA

GTTGCATGGTGCTGGTGCGCAGACCAATTTATGCCTACAGCCTCCTAATACAAAGACCTTTAACCTAATCTCCT

CCCCCAGCTCCTCCCAGTCCTTAAACACACAGTCTTTGAAGTAGGCCTCAAGGTCGGTCGTTGACATTGCTGGG

AGTCCAAGAGTCCTCTTATGTAAGACCTTGGGCAGGATCTGATGGGCGTTCACGGTGGTCTCCATGCAACGTGC

AGAGGTGAAGCGAAGTGCACACGGACCGGCAGATGAGAAGGCACAGACGGGGAGACCGCGTAAAGAGAGGTGCG

CCCCGTGGTCGGCTGGAACGGCAGACGGAGAAGGGGACGAGAGAGTCCCAAGCGGCCCCGCGAGGGGTCGTCCG

CGGGATTCAGCGCCGACGGGACGTAAACAAAGGACGTCCCGCGAAGGATCTAAAGCCAGCAAAAGTCCCATGGT

CTTATAAAAATGCATAGCTTTAGGAGGGGAGCAGAGAACTTGAAAGCATCTTCCTGTTAGTCTTTCTTCTCGTA

GACTTCAAACTTATACTTGATGCCTTTTTCCTCCTGGACCTCAGAGAGGACGCCTGGGTATTCTGGGAGAAGTT

TATATTTCCCCAAATCAATTTCTGGGAAAAACGTGTCACTTTCAAATTCCTGCATGATCCTTGTCACAAAGAGT

CTGAGGTGGCCTGGTTGATTCATGGCTTCCTGGTAAACAGAACTGCCTCCGACTATCCAAACCATGTCTACTTT

FIG. 13D

ACTTGCCAATTCCGGTTGTTCAATAAGTCTTAAGGCATCATCCAAACTTTTGGCAAGAAAATGAGCTCCT

CGTGGTGGTTCTTTGAGTTCTCTACTGAGAACTATATTAATTCTGTCCTTTAAAGGTCGATTCTTCTCAG

GAATGGAGAACCAGGTTTTCCTACCCATAATCACCAGATTCTGTTTACCTTCCACTGAAGAGGTTGTGGT

CATTCTTTGGAAGTACTTGAACTCGTTCCTGAGCGGAGGCCAGGGTCGGTCTCCGTTCTTGCCAATCCCC

ATATTTTGGGACACGGCGACGATGCAGTTCAATGGTCGAACCATGATGGCAAATTCTAGAATCGATAAGC

TTTTTGCAAAAGCCTAGGCCTCCAAAAAGCCTCCTCACTACTTCTGGAATAGCTCAGAGGCCGAGGCGG

CCTCGGCCTCTGCATAAATAAAAAAAATTAGTCAGCCATGGGGCGGAGAATGGGCGGAACTGGGCGGAGT

TAGGGGCGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCTGACTAATTGAGATGCAGATCTCGAGCT

AGCACGCGTAAGAGCTCGGTACCTCCCTAC

FIG. 14A
Nucleotide and deduced amino acid sequence of heavy chain of fusion
protein cDNA and protein encoded by tandem vector
(SEQ ID NO. 33)

```
atggaatgcagctgggtcatgctcttcctcctgtcaggaactgca
 M   E   C   S   W   V   M   L   F   L   L   S   G   T   A
ggtgtccattgccaggttcagctgcagcagtctggacctgagctg
 G   V   H   C   Q   V   Q   L   Q   Q   S   G   P   E   L
gtgaagcctggggctttagtgaagatatcctgcaaggcttctggt
 V   K   P   G   A   L   V   K   I   S   C   K   A   S   G
tacaccttcacaaactacgatatacactgggtgaagcagaggcct
 Y   T   F   T   N   Y   D   I   H   W   V   K   Q   R   P
ggacagggacttgagtggattggatggatttatcctggagatggt
 G   Q   G   L   E   W   I   G   W   I   Y   P   G   D   G
agtactaagtacaatgagaaattcaagggcaaggccacactgact
 S   T   K   Y   N   E   K   F   K   G   K   A   T   L   T
gcagacaaatcctccagcacagcctacatgcacctcagcagcctg
 A   D   K   S   S   S   T   A   Y   M   H   L   S   S   L
acttctgagaaatctgcagtctatttctgtgcaagagagtgggct
 T   S   E   K   S   A   V   Y   F   C   A   R   E   W   A
tactggggccaagggactctggtcactgtctctgcagctagcacc
 Y   W   G   Q   G   T   L   V   T   V   S   A   A   S   T
aagggcccatcggtcttccccctggcacctcctccaagagcacc
 K   G   P   S   V   F   P   L   A   P   S   S   K   S   T
tctgggggcacagcggccctgggctgcctggtcaaggactacttc
 S   G   G   T   A   A   L   G   C   L   V   K   D   Y   F
cccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagc
 P   E   P   V   T   V   S   W   N   S   G   A   L   T   S
ggcgtgcacaccttcccggctgtcctacagtcctcaggactctac
 G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y
tccctcagcagcgtggtgaccgtgccctccagcagcttgggcacc
 S   L   S   S   V   V   T   V   P   S   S   L   G   T
cagacctacatctgcaacgtgaatcacaagcccagcaacaccaag
 Q   T   Y   I   C   N   V   N   H   K   P   S   N   T   K
gtggacaagaaagttgagcccaaatcttgtgacaaaactcacaca
 V   D   K   K   V   E   P   K   S   C   D   K   T   H   T
tgcccaccgtgcccagcacctgaactcctggggggaccgtcagtc
 C   P   P   C   P   A   P   E   L   L   G   G   P   S   V
ttcctcttccccccaaaacccaaggacaccctcatgatctcccgg
 F   L   F   P   P   K   P   K   D   T   L   M   I   S   R
acccctgaggtcacatgcgtggtggtggacgtgagccacgaagac
 T   P   E   V   T   C   V   V   V   D   V   S   H   E   D
cctgaggtcaagttcaactggtacgtggacggcgtggaggtgcat
```

FIG. 14B

Nucleotide and deduced amino acid sequence of heavy chain of
fusion protein cDNA and protein encoded by tandem vector
(SEQ ID NO. 34)

```
     P   E   V   K   F   N   W   Y   V   D   G   V   E   V   H
     aatgccaagacaaagccgcgggaggagcagtacaacagcacgtac
     N   A   K   T   K   P   R   E   E   Q   Y   N   S   T   Y
     cgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaat
     R   V   V   S   V   L   T   V   L   H   Q   D   W   L   N
     ggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcc
     G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A
     cccatcgagaaaaccatctccaaagccaaagggcagccccgagaa
     P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E
     ccacaggtgtacaccctgcccccatcccgggatgagctgaccaag
     P   Q   V   Y   T   L   P   P   S   R   D   E   L   T   K
     aaccaggtcagcctgacctgcctggtcaaaggcttctatcccagc
     N   Q   V   S   L   T   C   L   V   K   G   F   Y   P   S
     gacatcgccgtggagtgggagagcaatgggcagccggagaacaac
     D   I   A   V   E   W   E   S   N   G   Q   P   E   N   N
     tacaagaccacgcctcccgtgctggactccgacggctccttcttc
     Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F
     ctctacagcaagctcaccgtggacaagagcaggtggcagcaggggg
     L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G
     aacgtcttctcatgctccgtgatgcatgaggctctgcacaaccac
     N   V   F   S   C   S   V   M   H   E   A   L   H   N   H
     tacacgcagaagagcctctccctgtctccgggtaaatcgagtatg
     Y   T   Q   K   S   L   S   L   S   P   G   K   S   S   M
     cactctgaccctgccgtcgaggtgagctgagcgtgtgtgacagt
     H   S   D   P   A   R   R   G   E   L   S   V   C   D   S
     attagtgagtgggtaacggcggcagacaaaaagactgcagtggac
     I   S   E   W   V   T   A   A   D   K   K   T   A   V   D
     atgtcgggcgggacggtcacagtccttgaaaaggtccctgtatca
     M   S   G   G   T   V   T   V   L   E   K   V   P   V   S
     aaaggccaactgaagcaatacttctacgagaccaagtgcaatccc
     K   G   Q   L   K   Q   Y   F   Y   E   T   K   C   N   P
     atgggttacacaaaagaaggctgcagggcatagacaaaaggcat
     M   G   Y   T   K   E   G   C   R   G   I   D   K   R   H
     tggaactcccagtgccgaactacccagtcgtacgtgcggcccctt
     W   N   S   Q   C   R   T   T   Q   S   Y   V   R   A   L
     accatggatagcaaaaagagaattggctggcgattcataaggata
     T   M   D   S   K   K   R   I   G   W   R   F   I   R   I
     gacacttcttgtgtatgtacattgaccattaaaaggtga 2448
     D   T   S   C   V   C   T   L   T   I   K   R   *
```

FIG. 15

Nucleotide and deduced amino acid sequence of light chain encoded by tandem vector
(SEQ ID NO. 35 and 36)

```
atggagacagacacactcctgctatggctcttgttgctcatgttt
 M   E   T   D   T   L   L   W   L   L   L   M   F
ccaggtaccagatgtgacatccagatgacccagtctccatcctcc
 P   G   T   R   C   D   I   Q   M   T   Q   S   P   S   S
ttatctgcctctctgggagaaagagtcagtctcacttgtcgggca
 L   S   A   S   L   G   E   R   V   S   L   T   C   R   A
agtcaggacattggtggtaacttatactggcttcagcagggacca
 S   Q   D   I   G   G   N   L   Y   W   L   Q   Q   G   P
gatggaactattaaacgcctgatctacgccacatccagtttagat
 D   G   T   I   K   R   L   I   Y   A   T   S   S   L   D
tctggtgtccccaaaaggttcagtggcagtaggtctgggtcagat
 S   G   V   P   K   R   F   S   G   S   R   S   G   S   D
tattctctcaccatcagcagccttgagtctgaagattttgtagac
 Y   S   L   T   I   S   S   L   E   S   E   D   F   V   D
tattactgtctacagtattctagttctccgtggacgttcggtgga
 Y   Y   C   L   Q   Y   S   S   S   P   W   T   F   G   G
gcgacaaagatggaaataaaacgaactgtggctgcaccatctgtc
 A   T   K   M   E   I   K   R   T   V   A   A   P   S   V
ttcatcttcccgccatctgatgagcagttgaaatctggaactgcc
 F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A
tctgttgtgtgcctgctgaataacttctatcccagagaggccaaa
 S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K
gtacagtggaaggtggataacgccctccaatcgggtaactcccag
 V   Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q
gagagtgtcacagagcaggacagcaaggacagcacctacagcctc
 E   S   V   T   E   Q   D   S   K   D   S   T   Y   S   L
agcagcaccctgacgctgagcaaagcagactacgagaaacacaaa
 S   S   T   L   T   L   S   K   A   D   Y   E   K   H   K
gtctacgcctgcgaagtcacccatcagggcctgagctcgcccgtc
 V   Y   A   C   E   V   T   H   Q   G   L   S   S   P   V
acaaagagcttcaacaggggagagtgttag
 T   K   S   F   N   R   G   E   C   *
```

FIG. 16

Nucleotide and deduced amino acid sequence of DHFR encoded by tandem vector

(SEQ ID NO. 37 and 38)

```
atggttcgaccattgaactgcatcgtcgccgtgtcccaaaatatg
M   V   R   P   L   N   C   I   V   A   V   S   Q   N   M
gggattggcaagaacggagaccgaccctggcctccgctcaggaac
G   I   G   K   N   G   D   R   P   W   P   P   L   R   N
gagttcaagtacttccaaagaatgaccacaacctcttcagtggaa
E   F   K   Y   F   Q   R   M   T   T   S   V   E
ggtaaacagaatctggtgattatgggtaggaaaacctggttctcc
G   K   Q   N   L   V   I   M   G   R   K   T   W   F   S
attcctgagaagaatcgacctttaaaggacagaattaatatagtt
I   P   E   K   N   R   P   L   K   D   R   I   N   I   V
ctcagtagagaactcaaagaaccaccacgaggagctcattttctt
L   S   R   E   L   K   E   P   P   R   G   A   H   F   L
gccaaaagtttggatgatgccttaagacttattgaacaaccggaa
A   K   S   L   D   D   A   L   R   L   I   E   Q   P   E
ttggcaagtaaagtagacatggtttggatagtcggaggcagttct
L   A   S   K   V   D   M   V   W   I   V   G   G   S
gtttaccaggaagccatgaatcaaccaggccacctcagactcttt
V   Y   Q   E   A   M   N   Q   P   G   H   L   R   L   F
gtgacaaggatcatgcaggaatttgaaagtgacacgttttttccca
V   T   R   I   M   Q   E   F   E   S   D   T   F   F   P
gaaattgatttggggaaatataaacttctcccagaatacccaggc
E   I   D   L   G   K   Y   K   L   L   P   E   Y   P   G
gtcctctctgaggtccaggaggaaaaaggcatcaagtataagttt
V   L   S   E   V   Q   E   E   K   G   I   K   Y   K   F
gaagtctacgagaagaaagactaa
E   V   Y   E   K   K   D   *
```

FIG. 17

FIG. 18

Fusion Protein

bind BBB HIR

bind trkB

FIG. 19

FIG. 20

SDS-PAGE

Reducing gel   Non-reducing gel
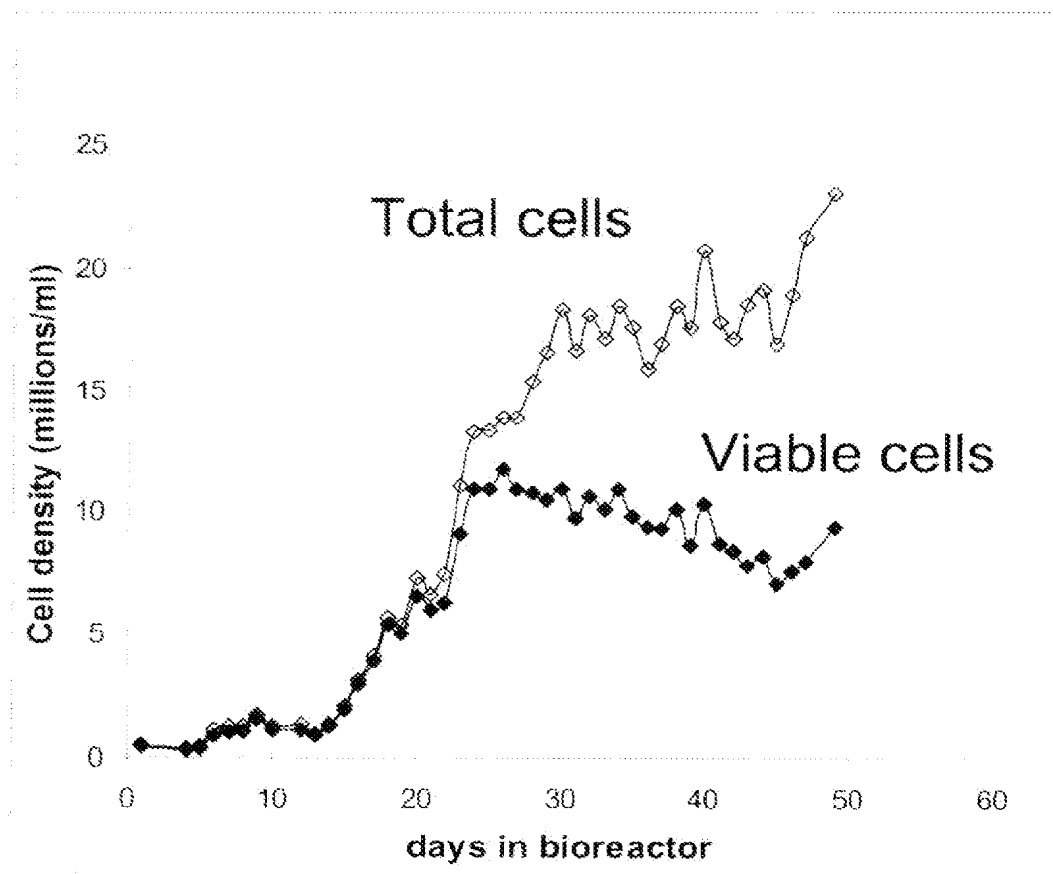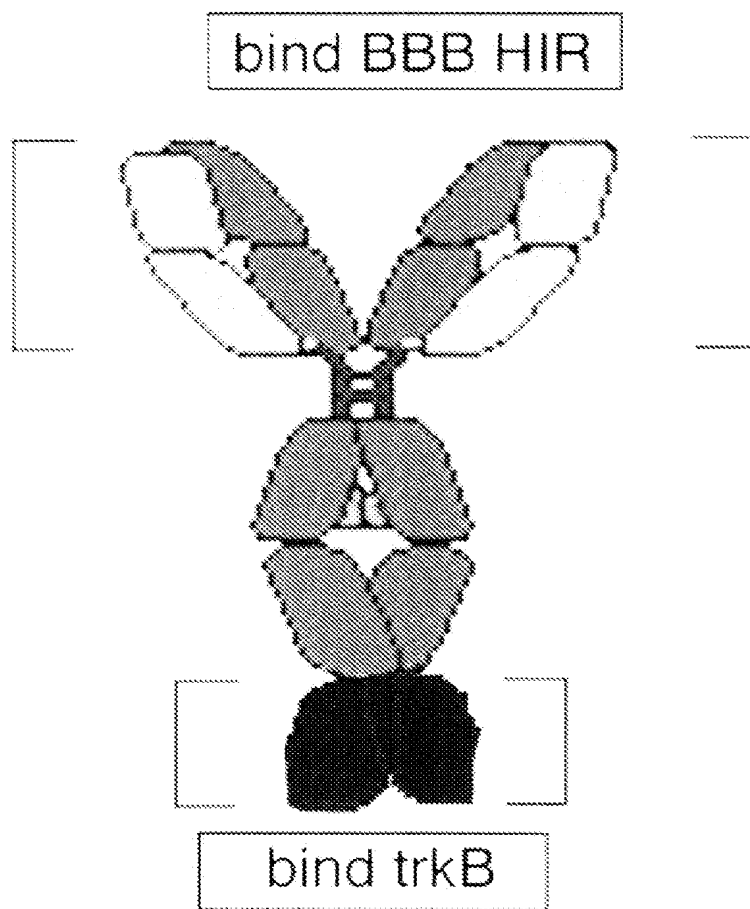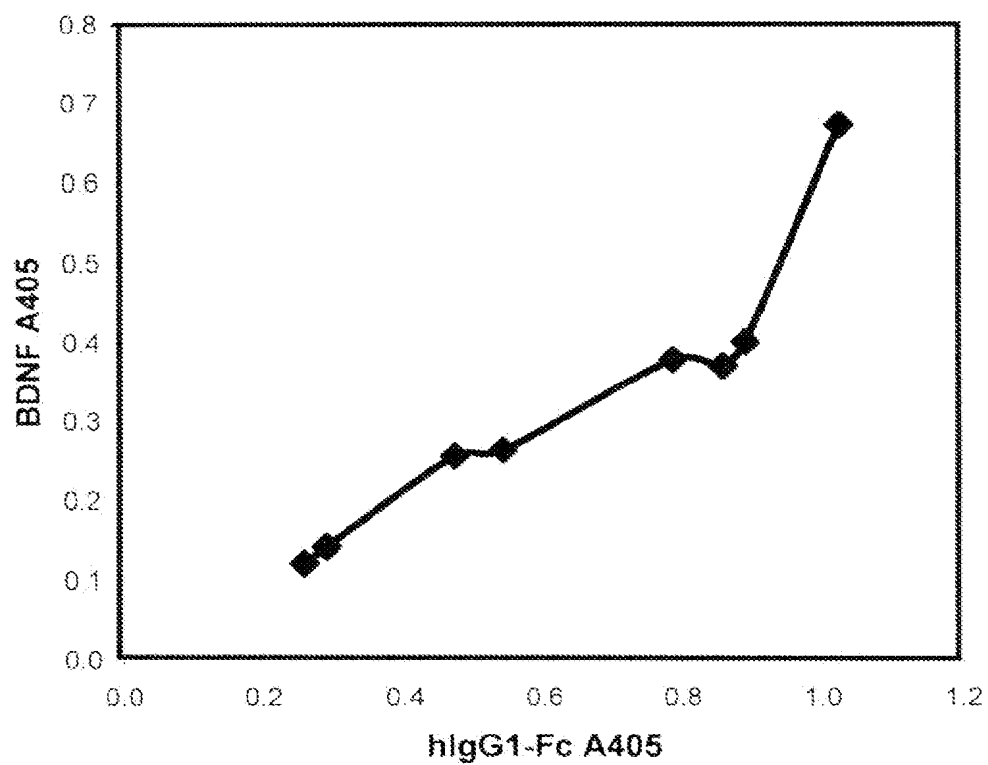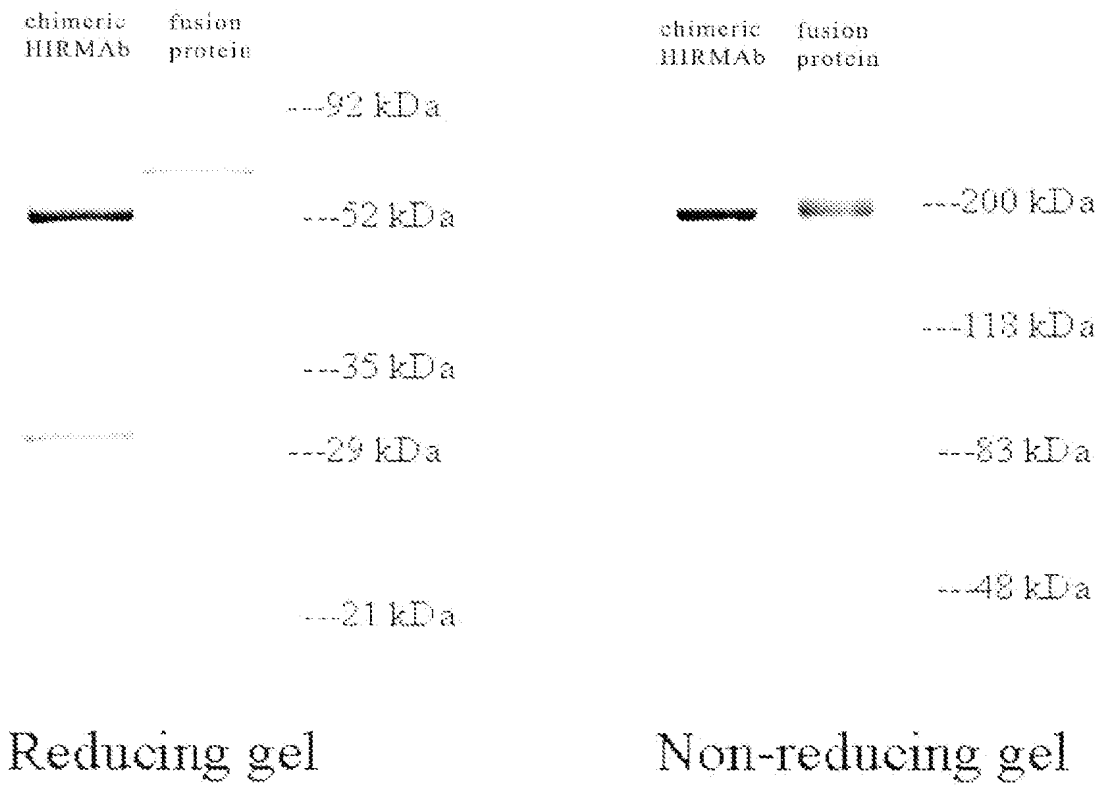
FIG. 21

FIG. 22

FIG. 23

FIG. 24

FIG. 25

A   Hypoxia-Reoxygenation Assay

SH-SY5Y human neural cells

↓

retinoic acid differentiation
to induce trkB

↓

16 hours of anoxia

↓

4 hours of
re-oxygenation, and
exposure to BDNF or
fusion protein

↓

MTT assay

FIG. 26

A

B   [3H]-fusion protein
      Radio-Receptor Assay
      Human Brain Capillaries

$K_D = 0.55 \pm 0.07$ nM
$B_{max} = 1.35 \pm 0.10$ pmol/mg$_p$
NSB $= 0.39 \pm 0.02$ pmol/mg$_p$

% Bound/mg protein

Fusion protein (µg/mL)

A plasma pharmacokinetics

C capillary depletion

B metabolic stability

D primate brain level

FIG. 28

FIG. 29

FIG. 30

FIG. 31

FIG. 32
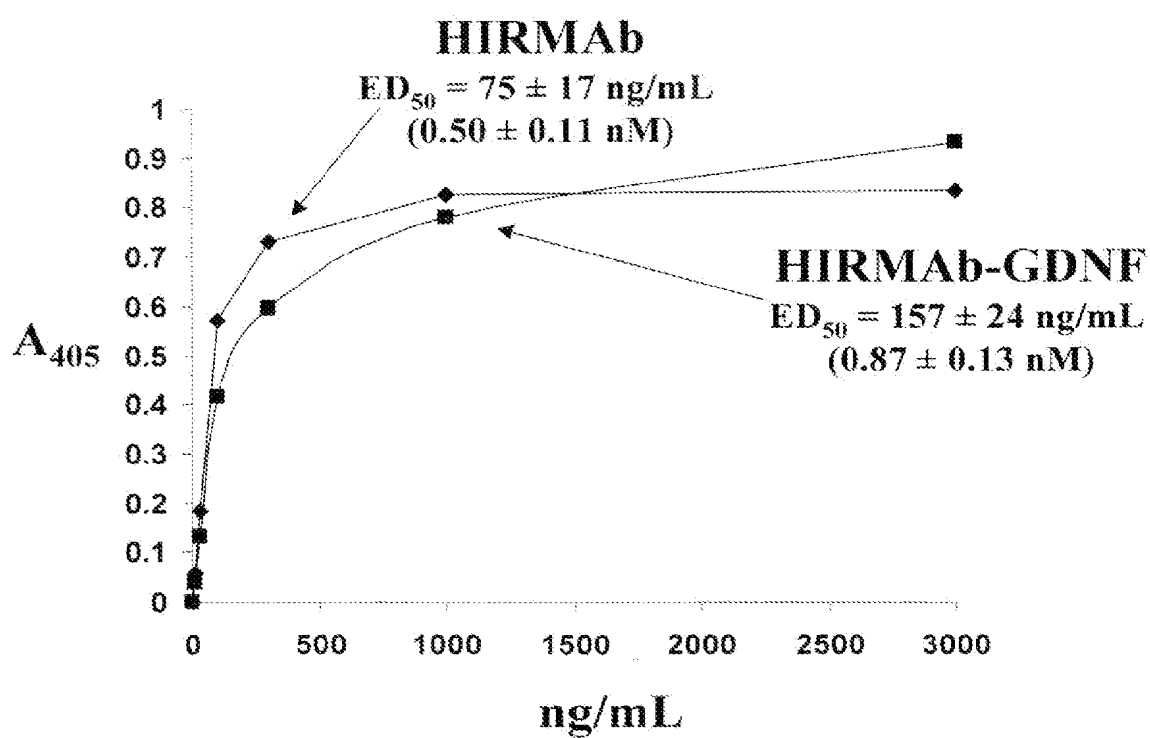
FIG. 33
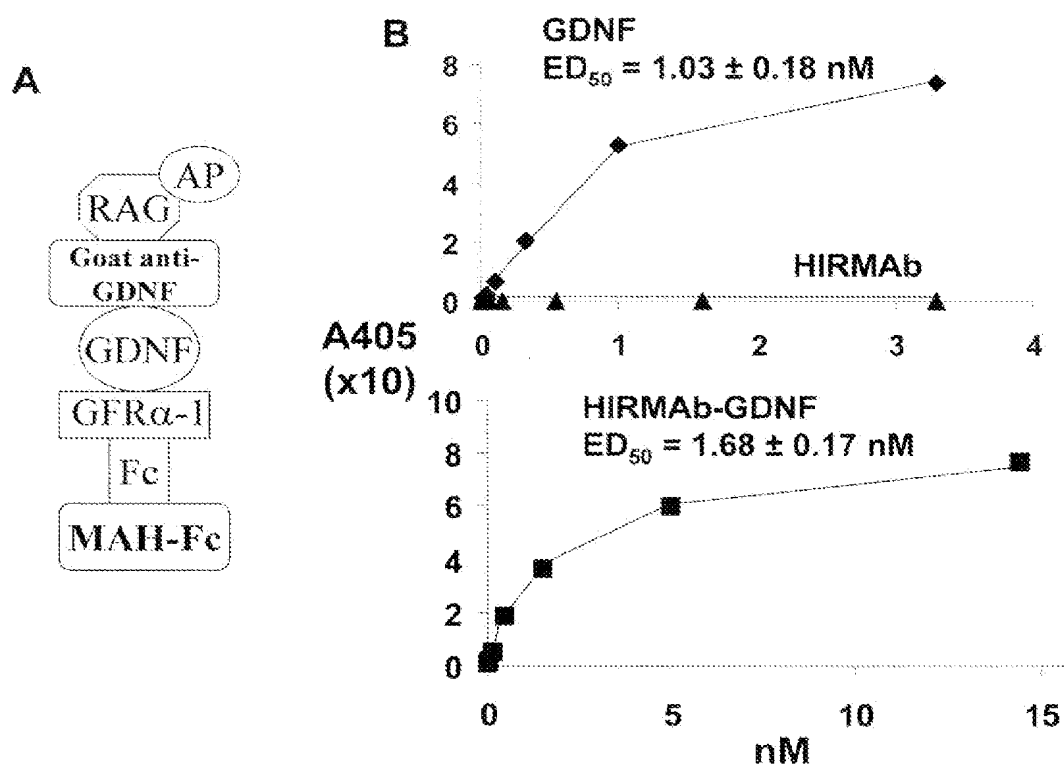
FIG. 34
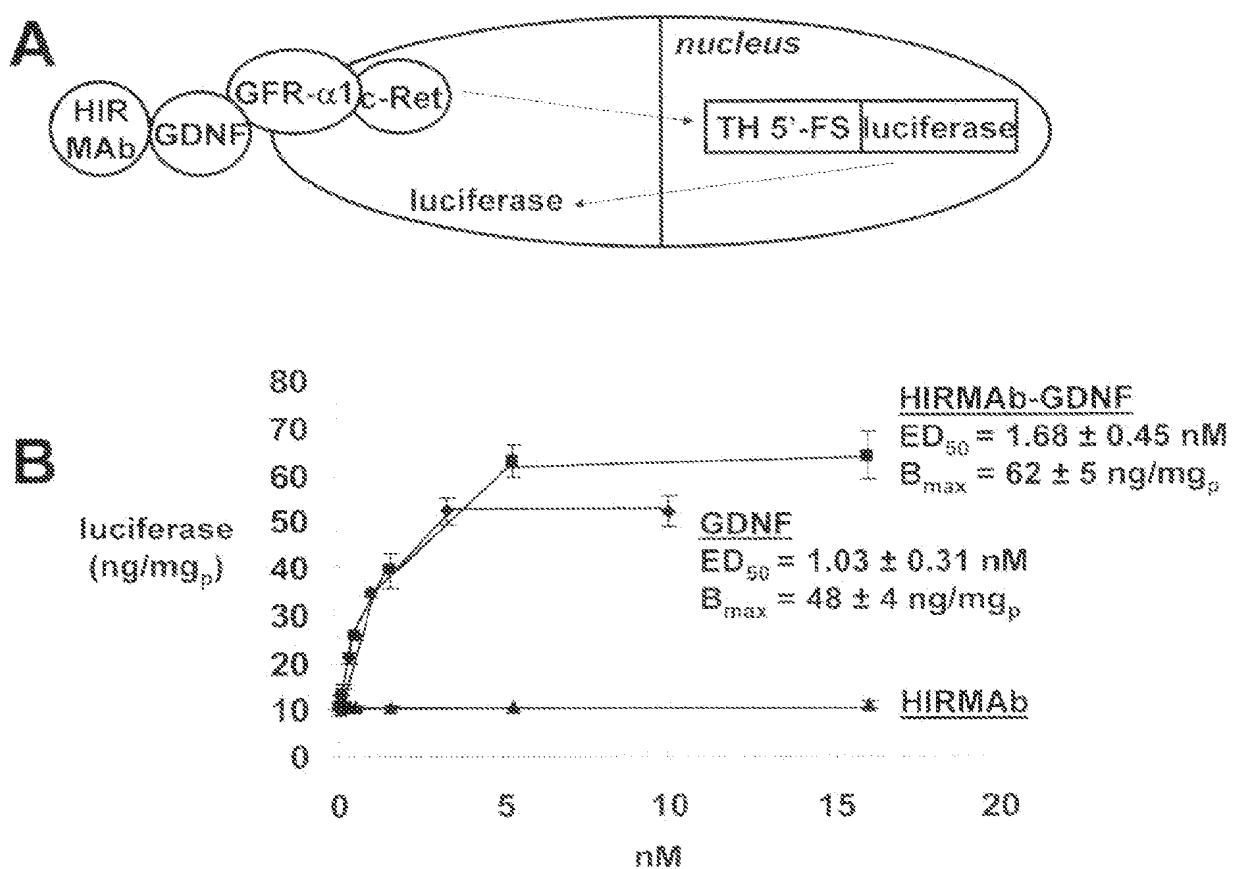
FIG. 35
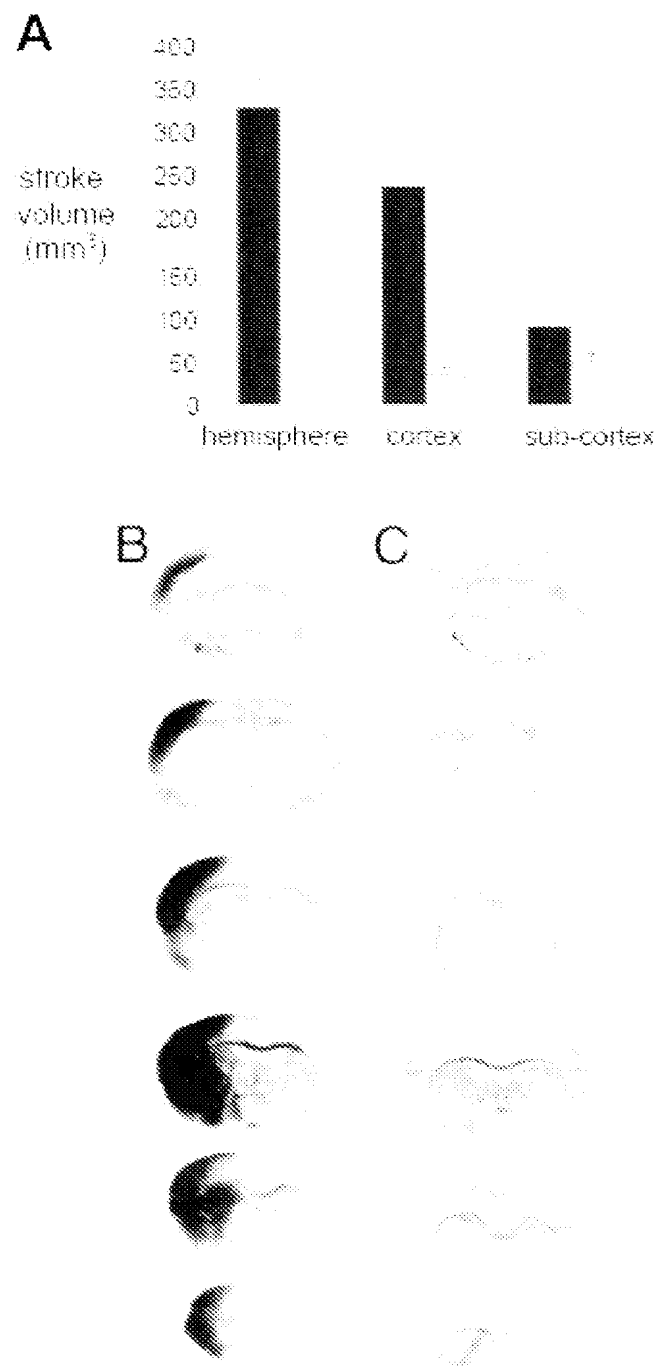
FIG. 36
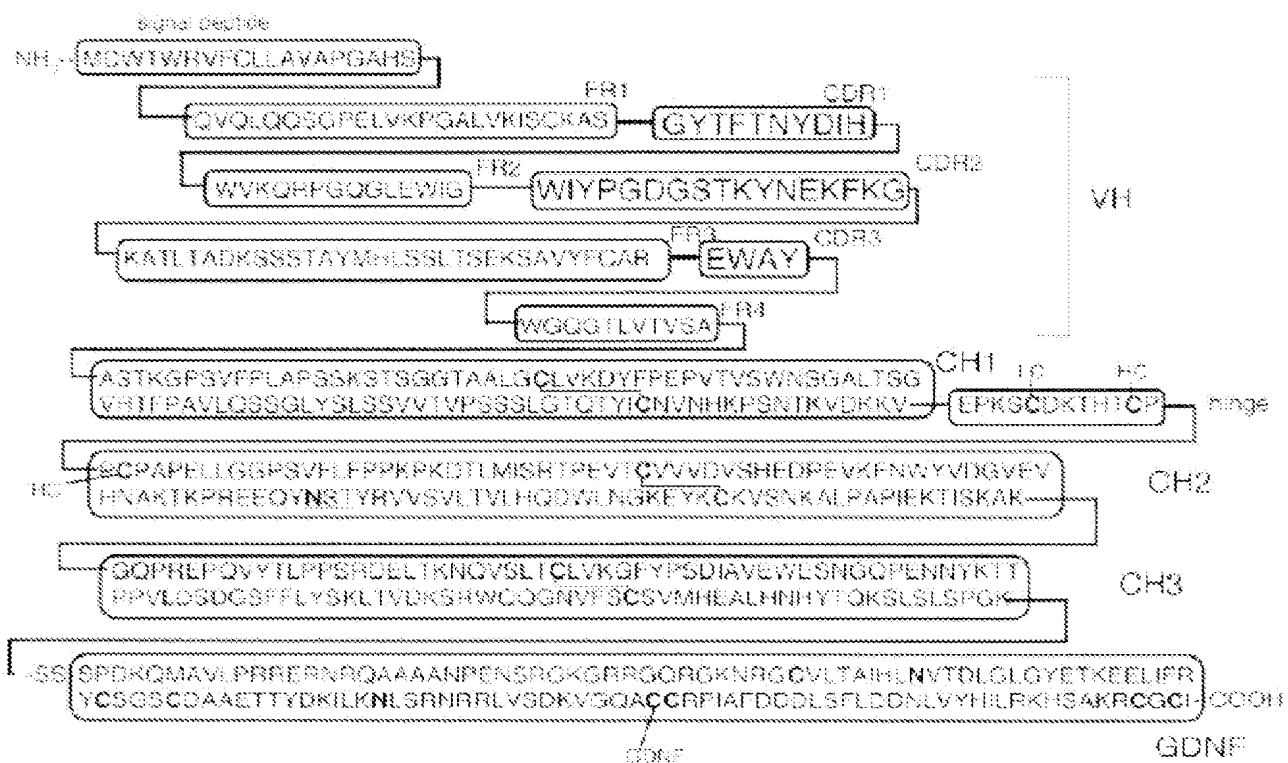
FIG. 37
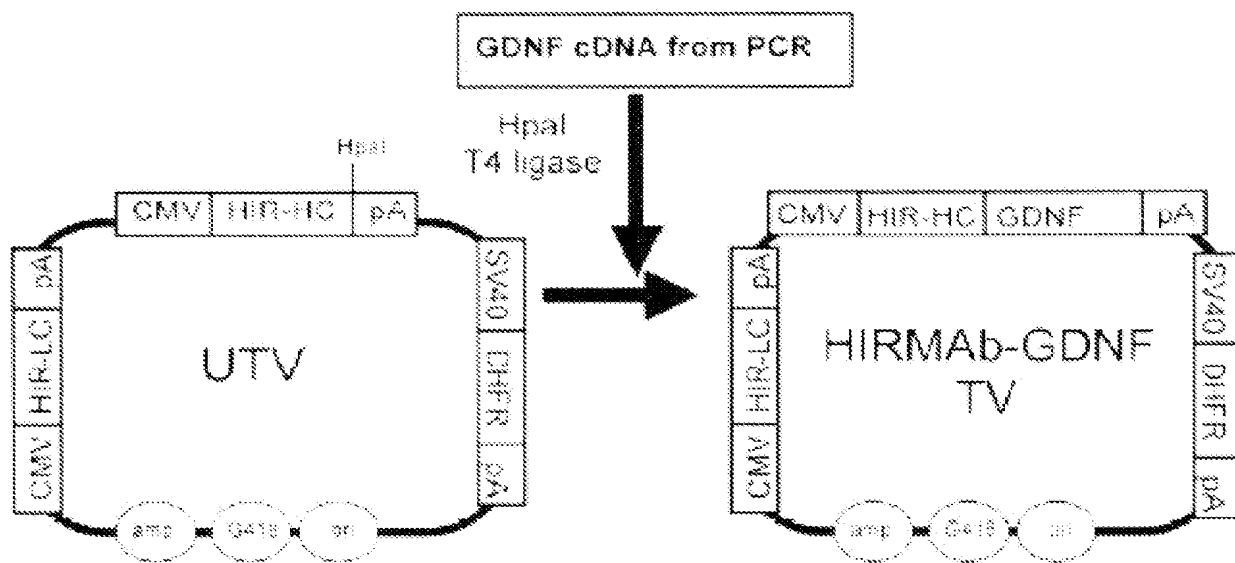
FIG. 38
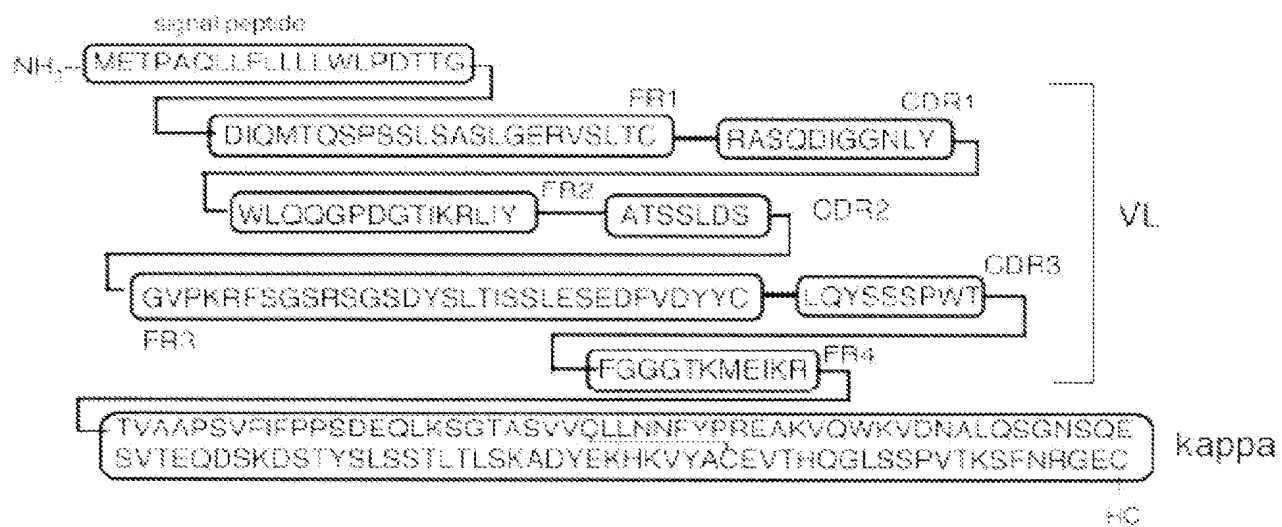
FIG. 39
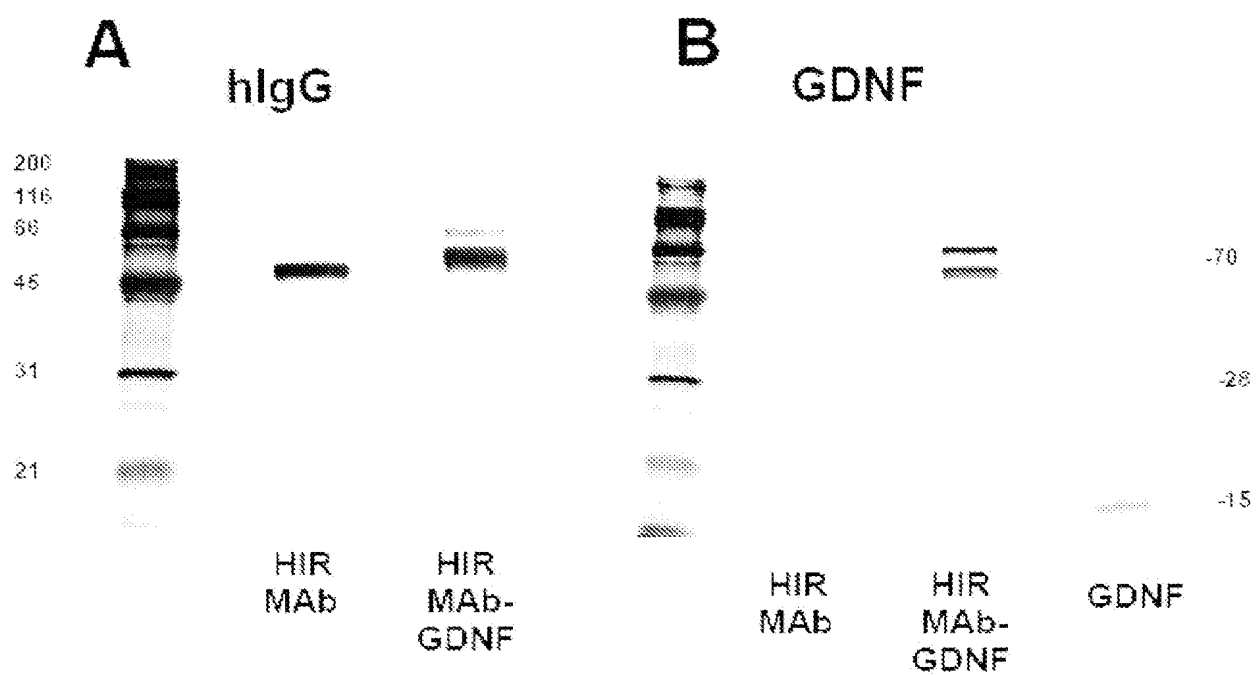
FIG. 40
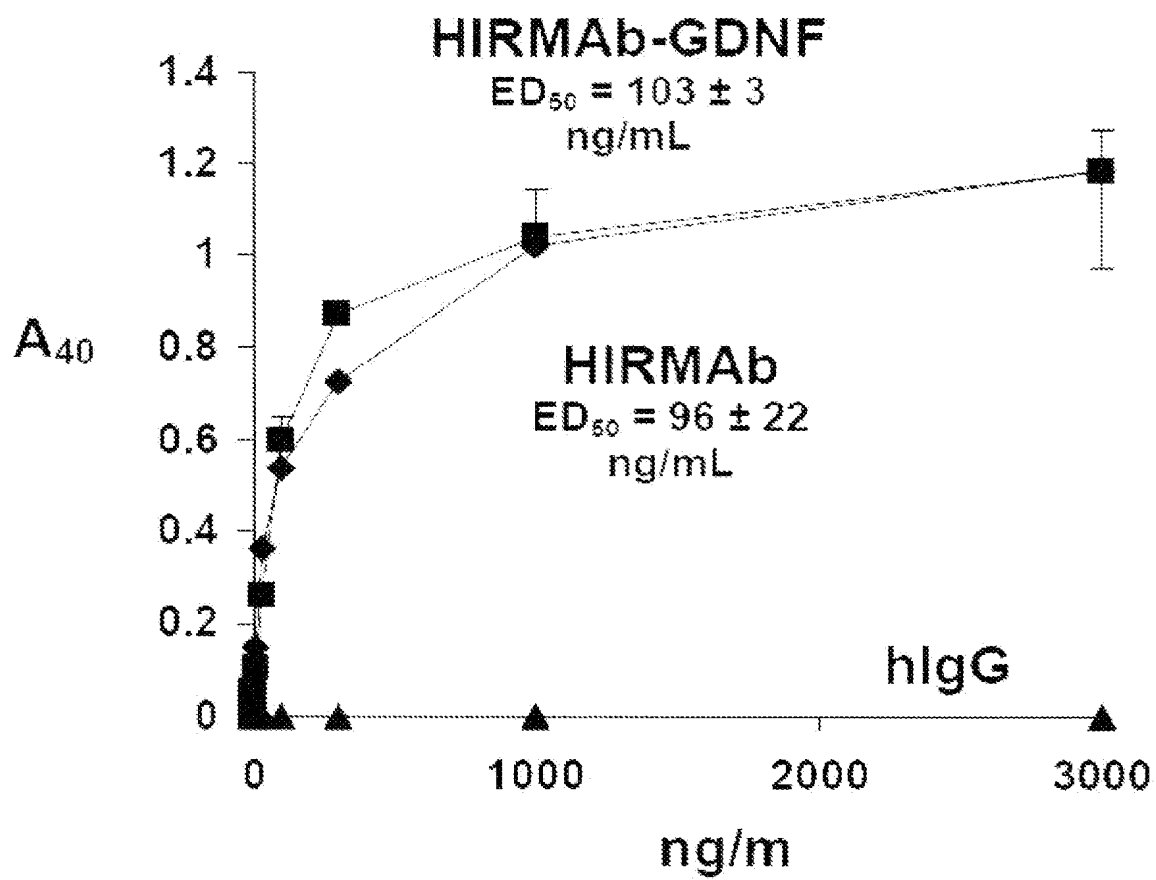
FIG. 41
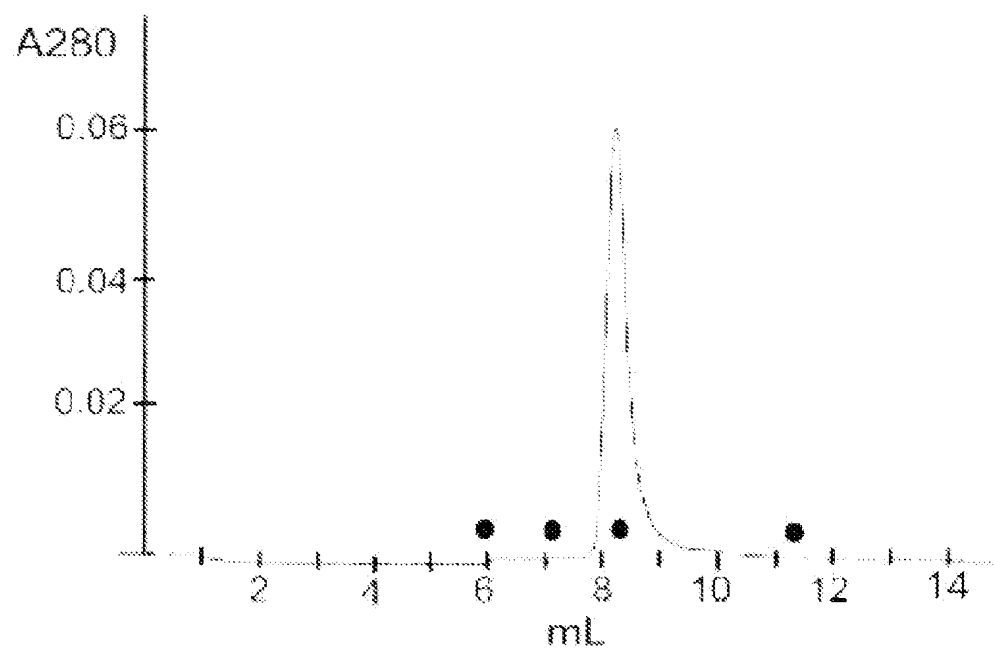
FIG. 42
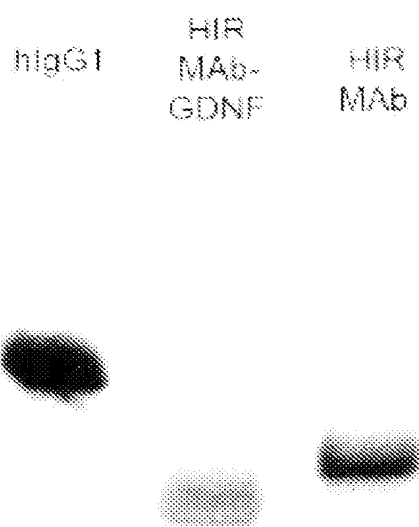
FIG. 43
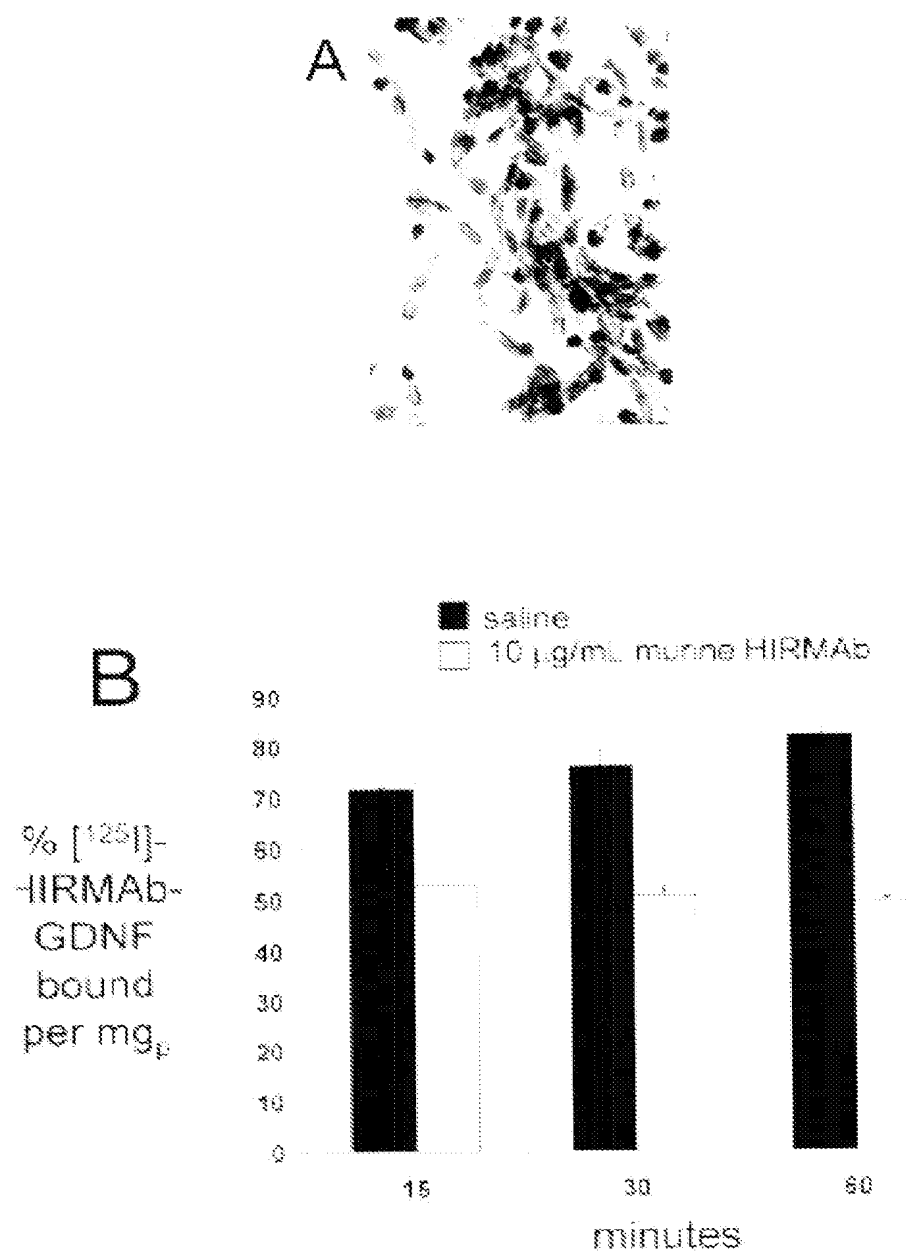
PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT
(PCT Article 17(2)(a), Rule 13ter.1(c) and (d) and 39)

Applicant's or agent's file reference 28570-706601	**IMPORTANT DECLARATION**	Date of mailing (day/month/year) **2 0 MAR 2009**
International application No. PCT/US08/84718	International filing date *(day/month/year)* 25 November 2008 (25.11.2008)	(Earliest) Priority date *(day/month/year)* 26 November 2007 (26.11.2007)

International Patent Classification (IPC) or both national classification and IPC

IPC: A61K 49/00(2006.01)

USPC: 424/9.1

Applicant
ARMAGEN TECHNOLOGIES, INC.

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:

   a. ☐ scientific theories.

   b. ☐ mathematical theories

   c. ☐ plant varieties.

   d. ☐ animal varieties.

   e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.

   f. ☐ schemes, rules or methods of doing business.

   g. ☐ schemes, rules or methods of performing purely mental acts.

   h. ☐ schemes, rules or methods of playing games.

   i. ☐ methods for treatment of the human body by surgery or therapy.

   j. ☐ methods for treatment of the animal body by surgery or therapy.

   k. ☐ diagnostic methods practised on the human or animal body.

   l. ☐ mere presentations of information.

   m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.

2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

   ☐ the description   ☐ the claims   ☐ the drawings

3. ☒ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:

   ☒ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

   ☒ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

   ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

4. ☐ A meaningful search could not be carried out without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-bis of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.

5. Further comments:
No response was filed to the ISA/225 mailed on 23 January 2009.

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